UNIVERSITY of EDINBURGH

STUDIES on BARLEY STARCH

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INTRODUCTION.

Starch is found very widely distributed in the plant kingdom. It forms the reserve carbohydrate material of most plants and is generally found stored in the seeds, bulbs or tubers. It constitutes one of the principal articles of diet, since it is hydrolysed, and, therefore, digested, relatively easily. Besides its use as a foodstuff, starch finds many applications in industry. It is not surprising that great interest has been shown in the chemistry and technology of this substance. Progress in elucidating its chemical structure, however, has been slow. Indeed, little satisfactory progress was made until after 1925, when more precise structural configurations were adopted for the simple sugars, such as glucose and maltose.

Our knowledge of the chemistry of starch is still far from complete. Studies have been confined, generally, to only a few of the commonly occurring starches, especially potato starch. Many starches have received little attention. It is surprising, however, that very little work has been published on the structure of barley starch. This substance may be regarded as the starting material for the series of processes in brewing which result in the production of alcohol by fermentation. A complete understanding of these processes is dependent, therefore, on our knowledge of the chemistry of barley starch.

The object of this study is therefore twofold; firstly to compare the chemical properties of barley starch with those of the starches already studied, and secondly to attempt to solve

those problems in the chemistry of starch which are unsettled at present.

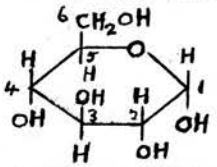
THE CHEMISTRY OF STARCH.

Starch occurs in nature in a granular form. The size and shape of the granules are characteristic of the source from which they have been obtained. The granules are insoluble in cold water, but disperse in hot water to form a colloidal gel. As early as 1814 it was recognised that the granules are composed, almost entirely, of polysaccharide material which on acid hydrolysis yields glucose (1). Small amounts of other substances are normally found associated with native starch. These substances include phosphorus (2), fatty acids (3) and protein matter (4).

Schoch (5) has shown recently that the fatty acids are merely adsorbed on the carbohydrate and may be removed by extraction with water-soluble fat solvents, such as methanol and dioxane. Schoch has also found that the phosphorus content of cereal starches may be reduced by treatment with these solvents. The phosphorus content of potato starch is not reduced by solvent extraction, and thus appears to be chemically linked to the starch (2). The protein content of starch is generally very small, and is believed to be a residue from the enzymes originally responsible for the synthesis of the starch (4). It is now generally accepted that these substances have no significance in connection with the general chemical structure of the starch molecule.

D - Glucose (fig. 1) is the only monosaccharide which has been conclusively shown to be present in starch. The yield of glucose, estimated by polarimetric and reducing power measurements, and by isolation of crystalline glucose, is

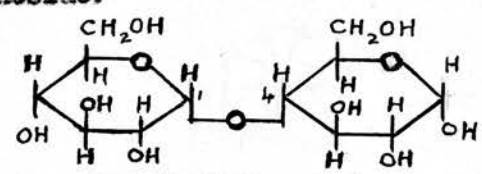
practically quantitative. If the acid hydrolysis of



(FIG. I)

starch is not allowed to reach completion, maltose may be found among the hydrolysis products. This disaccharide was isolated in early experiments by O'Sullivan (1872-79) (6) as the principal yield from starch which had been submitted to the action of β-amylase preparations. The possibility existed, however, that the maltose was not a breakdown product and, therefore, an integral part of the starch molecule, but simply a product of synthesis from the secondary reaction of the hydrolysing agents. Karrer (7) treated starch with acetyl bromide and obtained heptaacetyl maltosido-1- bromide. The latter readily yielded heptaacetyl maltose, thus providing chemical evidence that maltose could be obtained from starch.

These facts provided the first clues as to the way in which glucose residues were joined together in the starch molecule. It was not until 1926, however, that these discoveries developed their full significance. In that year, Irvine and Black (8), and Cooper, Haworth and Peat (9) showed that the structure of maltose (fig. II) was 4-(α-D-glucopyranosido)-D-glucopyranoside.



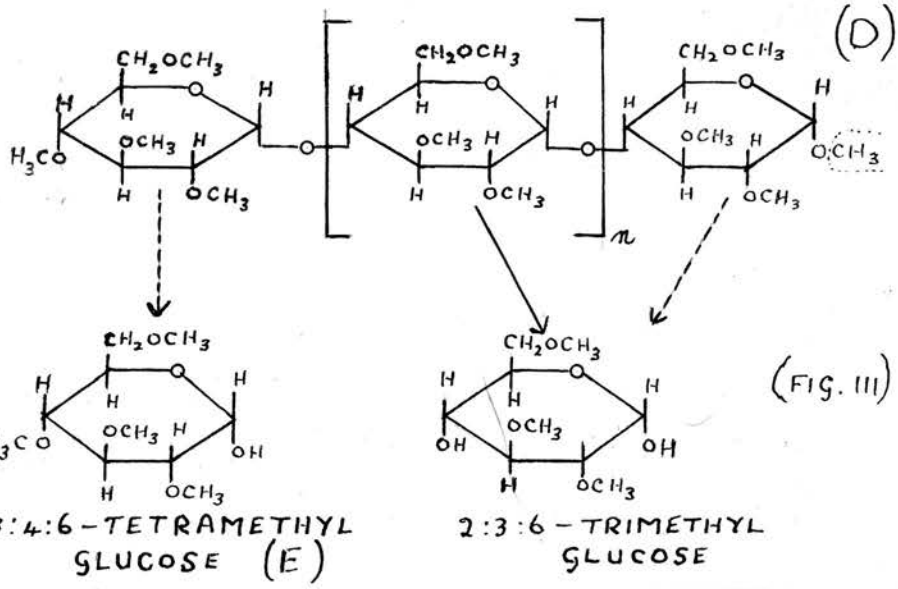
(FIG. II)

Thus it was suggested that the glucose residues in starch were present in the pyranose form and were joined by 1:4-α-linkages as in the maltose structure, a view which was supported

by Haworth, Hirst and Webb (10) who showed that starch could be methylated to a trimethyl derivative which on acid hydrolysis gave 2:3:6- trimethyl glucose (fig. III) in over 90% yield. Proof that two contiguous glucopyranose units, joined as in maltose, existed in the starch molecule was obtained by Haworth and Percival (11). By the acetolysis of methylated starch (see fig. IV) at room temperature, followed by oxidation, methylation and separation by distillation in a high vacuum, methyl octamethyl maltobionate (A - fig. IV) was obtained.

This ester on hydrolysis yielded 2:3:4:6- tetramethyl glucopyranose (B) and 2:3:5:6- tetramethyl γ - gluconolactone (C), which were the same products obtained from a similar experiment with maltose (9). Further evidence of the presence of continuous α- linkages within the chains of glucose units was provided by Freudenberg's studies on optical rotatory power (12). In the case of starch, agreement between the theoretical and experimental results was sufficiently close to conclude that only one linkage in 30 (possibly 50 or 60) could deviate from the uniform α- linkage.

It was apparent, however, that, if the chains of glucopyranose units were finite in length, two end-glucose units should be distinguishable in the methylated derivative. The first, the 'reducing' end-group (D, fig. III) on hydrolysis will yield 2:3:6- trimethyl D-glucose, since the methoxyl group on C_1 is glucosidic in character and removed by dilute acids. The second, the non-reducing end-group, will exist, however, as 2:3:4:6- tetramethyl D-glucose (E, fig. III) after

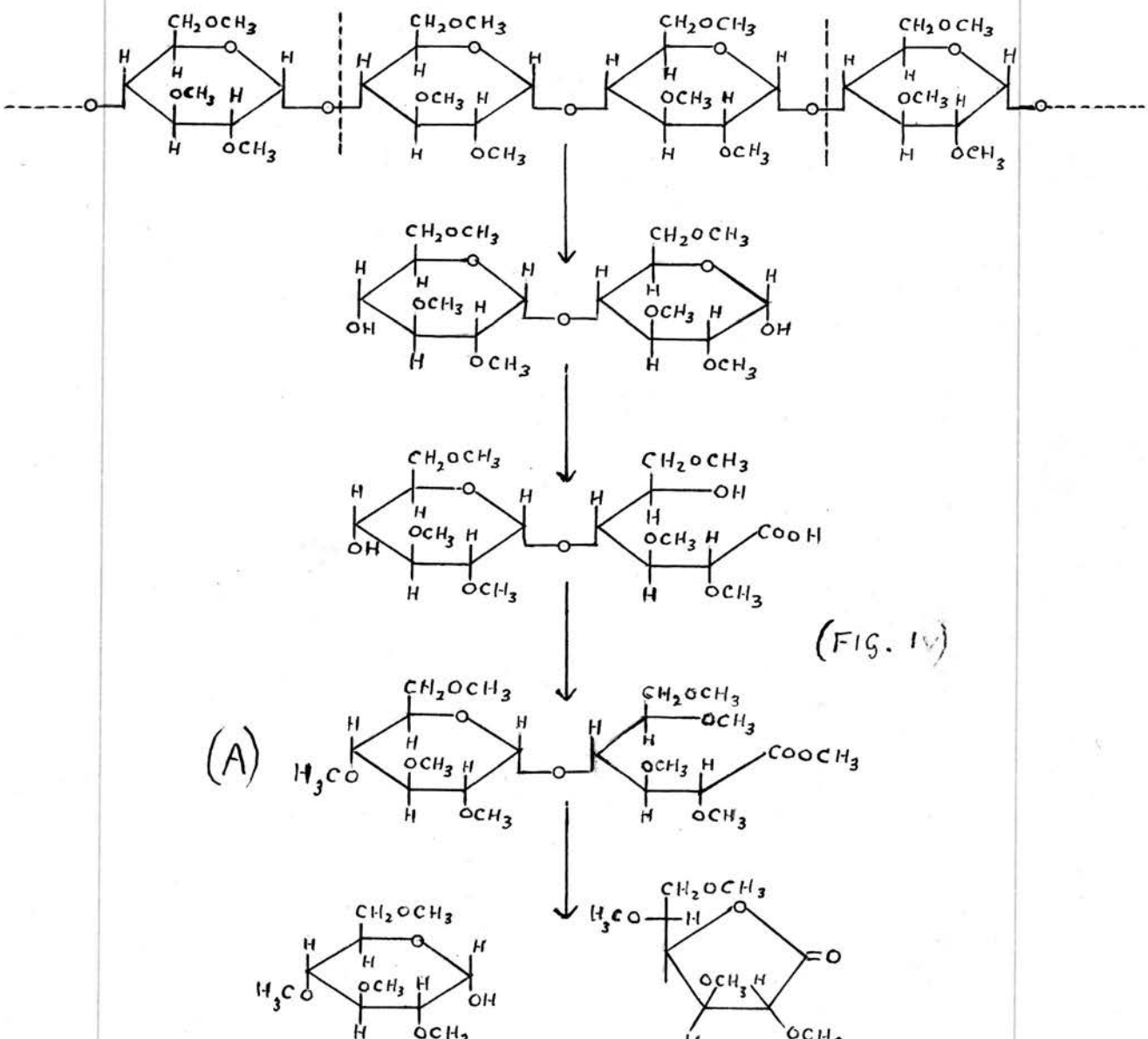


(D)

(FIG. III)

2:3:4:6-TETRAMETHYL GLUCOSE (E)

2:3:6-TRIMETHYL GLUCOSE



(FIG. IV)

(A)

2:3:4:6-TETRAMETHYL GLUCOSE

(B)

2:3:5:6-TETRAMETHYL γ-GLUCONOLACTONE

(C)

hydrolysis. Estimation of this component will allow the length of the chain to be calculated.

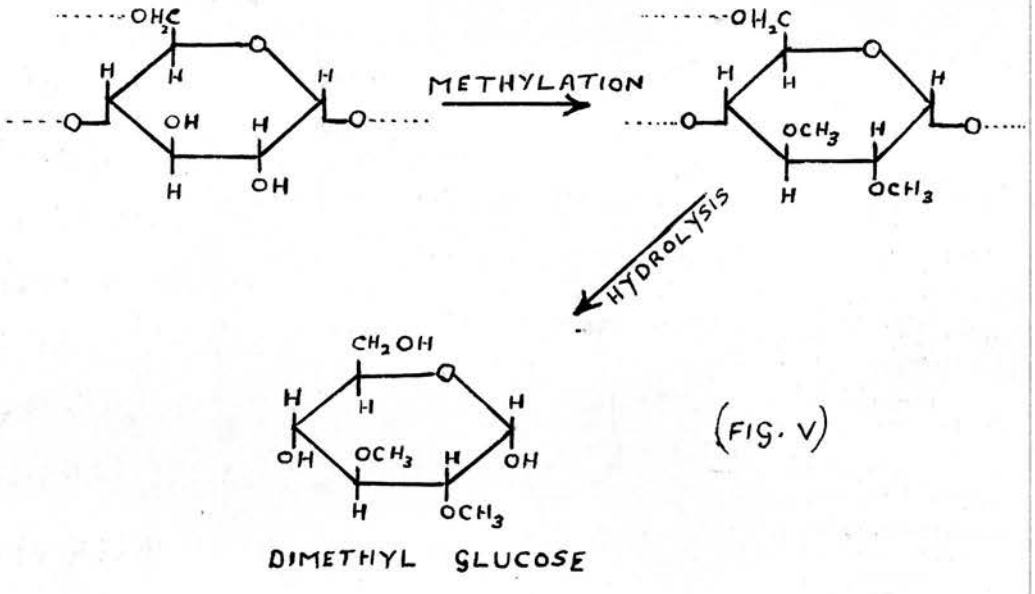
Examination of the hydrolysis products of methylated potato starch by Hirst, Plant and Wilkinson (13) revealed the presence of 4-5% of 2:3:4:6- tetramethyl D-glucose corresponding to a chain length of some 25 glucose units. Studies on the methylated derivatives of other starches (Table I)

TABLE I.

Source of Starch	"Chain length" calc. from yield of tetramethyl glucose	Reference
Acorn	30	14
Banana	24	15
Canna	27	16
Horse chestnut	28	17
Maize (Golden Bantam)	25	18
Rice	30	19
Waxy Maize	26-30	20
Wheat	24	17

showed the presence of similar amounts of 2:3:4:6- tetramethyl D-glucose and of corresponding chain lengths.

The simplest conclusion from these facts would be that the starch molecule consisted of a straight chain of 24-30 units, corresponding to a molecular weight of 4,000-5,000. It was soon recognised, however, that this structure was incorrect since starch was shown to have a molecular weight greatly in excess of 5,000. Determinations (19, 21) on solutions of undegraded methylated starch showed that the observed molecular weight was of the order of 5×10^5. The presence of dimethyl D-glucose (fig. V) (4% or greater) in the hydrolysis



(FIG. V)

products of all methylated starches examined, seemed to indicate the existence of a branched and more complicated structure.

It gradually became apparent, too, that all the physical and chemical properties of starch could not be explained on the basis of a single chemical structure. Suggestions had been put forward, from time to time, that starch was not chemically homogeneous. Various workers reported the isolation of two components. In the light of present knowledge, the work of Maquenne (22) in 1905 stands out. He isolated a component from starch which could be completely hydrolysed to maltose by the action of β-amylase. To this component, the name amylose was subsequently given. Maquenne suggested also that a second component existed which was not completely hydrolysed by β-amylase to maltose, but gave rise to a limit dextrin. This component he named amylopectin. These results and suggestions of Maquenne have been shown to be correct by the work of the past few years. Amylopectin is now accepted as that component of starch which is incompletely hydrolysed by β-amylase, and

whose solutions give a reddish-purple coloration with iodine. Amylose, on the other hand, is the component which is completely hydrolysed by β-amylase; its solutions give a deep blue coloration with iodine.

In the interval between 1905 and 1940, however, Maquenne's results were ignored. Different authors substituted other definitions for the terms amylose and amylopectin.

The discovery by Hanes (23) of a "synthetic starch" in 1940 effected a renewal of interest in the possible fractionation of starch. The "synthetic starch", although similar in appearance to ordinary starch, differed from it in many properties (see later). Thus the question arose as to whether this new variety of starch was purely a product of laboratory synthesis or was a normal component of natural starch. Attempts to settle this question resulted in the discovery of new methods of fractionation. Although not completely successful, they were sufficient to establish the heterogeneous nature of starch conclusively. The two main components, amylose and amylopectin, were obtained in a sufficient degree of purity. Much progress has been made in elucidating their chemical structures. Whether they exist in starch independently of one another or combined in some form of molecular complex is not yet known, however.

THE FRACTIONATION OF STARCH.

The successful fractionation of starch depends upon the complete dispersion of the starch granule by methods which will not cause physical or chemical degradation. It will be realised that this is difficult to obtain, especially as

retrogradation (or precipitation) of starch from solution takes place readily under certain conditions. The failure of earlier workers to conform, as closely as possible, to conditions for the dispersion of starch without degradation has probably resulted in progress being so slow and confused. Many of the fractions obtained in this early period were undoubtedly degraded, and many were mixtures which only varied in physical properties.

Little need be said, therefore, about the methods used by the earlier workers. Although Maquenne did succeed in reaching satisfactory conclusions by present day standards, his method of fractionation, which involved the retrogradation of the more unstable amylose component from solution, has since been found to be unsatisfactory.

Samec (24) in 1920 succeeded in obtaining a fractionation of potato starch, using an electrophoretic method. This depended on the presence of non-carbohydrate material, namely, organically-bound phosphate, which was present in only the amylopectin component and imparted polarity to it. As Schoch has pointed out (p. 2), the phosphorus content of cereal starches is greatly reduced by solvent extraction. Thus small polarity will be imparted to the amylopectin fractions of these starches, and fractionation will not be effected.

Baldwin (25) in 1930 used a method which was applicable to most starches. This involved leaching of the swollen but intact starch granules with hot water. The aqueous extract yielded the more soluble amylose fraction, leaving the

amylopectin as the insoluble residue. The method, however, requires repeated extractions, during which it is difficult to maintain perfectly sterile conditions, and the amylose tends to retrograde from solution. Meyer (26) utilised this method in 1940 and obtained relatively pure samples of amylose and amylopectin. Meyer was able to make a study of the physical and chemical properties of these components.

One of the most important contributions to the problem of obtaining pure starch fractions was made by Schoch (27) in 1942. His work was based on an observation of Alsberg (28) that autoclaved starch solutions gave a crystalline precipitate with alcohol. Schoch dispersed fat-free starch in an aqueous solution saturated with n-butanol by autoclaving at 120°C. The resulting solution was allowed to cool very slowly. The amylose component separated out as a micro-crystalline complex containing butanol which could readily be separated by centrifuging. The complex contained amylopectin as impurity, but this was readily removed. The complex was found to be readily soluble in warm water. By resaturating with butanol, and cooling slowly, reprecipitation or "recrystallisation" of the amylose complex took place, leaving the amylopectin impurity in solution. After concentration of the mother liquors, the amylopectin fraction was obtained by precipitation with water-soluble alcohols.

Various workers (29-32) quickly recognised that the amylose was capable of forming an insoluble complex with polar compounds, such as alcohols, which possess the property of forming hydrogen

bonds. Amylopectin, on the other hand, either did not form such a complex or formed one which was soluble. Examples of the polar compounds, which have been utilised, included primary alcohols (29), pyridine (30,31), nitroparaffins (31), especially the nitropropanes, thymol (32) and carboxylic acids (31).

Although the general method of fractionation by precipitation with polar organic solvents is that most generally applied at present, attention has also been paid to the method of "selective adsorption". Various workers (33,34) have claimed that when starch pastes were treated with adsorbents, principally cotton, the amylose was preferentially bound. Although this method involved no chemical degradation, the amounts of amylose adsorbed were very small and rendered the method impracticable.

Bourne, Donnison, Peat and Whelan (35) have recently developed a method whereby the starch was adsorbed on aluminium hydroxide precipitated from an aqueous solution. When the precipitate was boiled with water, the amylose fraction was found to pass into solution, whereas little or none of the amylopectin dissolved. If less aluminium hydroxide were used, the amylopectin was preferentially adsorbed, leaving the amylose fraction in solution. It was found, however, that yields of the amylose fraction were often low. The ash values of the fractions tended to be much higher (1-2%) than that generally found for starch fractions prepared by other methods (approx. 0.2%).

The success of Schoch's fractionation procedure allowed progress to be made in two different spheres. Firstly, the fractions were available for purely structural studies, and secondly, for analytical studies, since the precipitation of amylose appeared to be almost quantitative. It will be readily appreciated that, as the earlier workers did not obtain sufficiently pure samples of the components, they were not able to develop satisfactory analytical methods. Without these, progress was bound to be slow and confused.

Bates, French and Rundle (36) in 1942-3 utilised starch fractions prepared by the method of Schoch to develop a method for determining the relative proportions of amylose in mixtures and in whole starch. The method was based on the relative affinities of amylose and amylopectin for iodine. It has already been mentioned (p. 8) that solutions of these fractions give different colorations with iodine. These workers showed that with amylose the iodine is firmly bound as the result of complex formation, whereas with amylopectin the iodine appeared to be colloidally adsorbed. It was also noted that the iodine is adsorbed preferentially by the amylose fraction, and not until complex formation with it is complete does the amylopectin commence to adsorb iodine. Bates, French and Rundle were able, therefore, to develop a potentiometric method which enables the amount of iodine firmly bound by the starch or by samples of crude amylose and amylopectin to be evaluated. Thus it was readily assumed that the sample which binds the greatest proportion of iodine, under given conditions, represents the

purest fraction of amylose. On this basis, the amylose content of any sample may be calculated.

Schoch (29) has pointed out that it is important that no other molecules are present which are capable of being adsorbed on the starch since they will prevent the full adsorption of iodine (37). This is particularly true of the fatty acids which are normally associated with cereal starches. These must be removed, first of all, by extraction with water-miscible organic solvents (see p. 2).

It will be noted that the behaviour of whole starch during potentiometric titration with iodine constitutes a powerful argument in favour of the theory that there are only two components in starch.

Kerr and Severson (38) applied a butanol precipitation to the hot water extract of corn and tapioca starches. By this combination of fractionation methods, amylose fractions were obtained with exceptionally high iodine adsorptions. Thus the corn and tapioca amyloses adsorbed, respectively, 20.5% and 20.7% of their own weight of iodine. Higginbotham and Morrison (39) have subjected various starches to precipitation with butanol and pyridine. They have found that the weight of iodine adsorbed by the amylose fractions of each of the starches reaches a constant value of 21.5% after several reprecipitations. This represents the highest uptake of iodine reported for any amylose fraction. In the Experimental section of this Thesis, in assessing the contents of the various fractions, it has been assumed that pure amylose takes up under the standard conditions

of Bates, French and Rundle (loc. cit.) modified by Hudson, Schoch and Wilson (40), 21.5% of its own weight of iodine.

Two main uses have been made of the potentiometric iodine titration method. Firstly, the amylose content of native starches has been examined. Brown, Halsall, Hirst and Jones (41) have shown (Table II) that amylose is generally present to the extent of 15-25% in the commonly occurring starches.

TABLE II.

Source of Starch	Amylose Content (%)
Arrowroot	20
Banana	21
Maize	23
Pearl Manioc	16
Potato	18
Rice I	14
Rice II	15
Sago	26
Sweet Potato	18
Tapioca	19
Wheat	19

Certain so-called "waxy" starches, which have been previously known to give a red colour with iodine, were found to contain less than 2% amylose (36,41,42). These starches may prove of considerable value since they may be considered as natural amylopectins. Only one sample of starch has been shown to contain only amylose. This has been found by Peat, Bourne and Nicholls (43) in the "Steadfast" variety of wrinkled pea. Other varieties of wrinkled pea starch have been found to possess 50-60% of amylose (44).

Secondly, the potentiometric iodine titration method has been of immense value in comparing the relative efficiences of the different fractionation methods. The development of a

colorimetric method of analysis by Hassid and McCready (45) in 1943 assisted this work. These workers also utilised the reaction of the starch components with iodine. Under standard conditions laid down by Hassid and McCready, the amylose fraction gives a much more intense coloration with iodine than the amylopectin fraction. Mixtures of the two produce colorations which are intermediate in intensity between that of amylose and amylopectin. By quantitatively determining the colour intensity of a mixture, the proportion of amylose and amylopectin can be estimated. Bourne, Haworth, Macey and Peat (46) have modified the conditions used by Hassid and McCready very slightly. They have also designated the quantitative reading of the colour intensity of the carbohydrate-iodine complex, measured by the "Spekker" photoelectric absorptiometer, by the term "Blue Value". Higginbotham and Morrison (47) have found that the "Blue Values" of the amylose fractions from several different starches, which adsorb the same proportion of iodine by potentiometric titration, varied between 1.26 and 1.48. The "Blue Values" of the corresponding amylopectins varied between 0.06 and 0.16. In spite of the inability of the colorimetric method to give consistent values for the pure fractions, it is much simpler in operation and enables much speedier, although less accurate, evaluation of the amylose content than the potentiometric titration method.

Schoch (29), after his initial success with butanol, evaluated the fractionating efficiences of many other alcohols. It must be pointed out, however, that it is, in many cases,

difficult to assign relative fractionation efficiencies since the purity and recovery of each of the fractions must be considered independently. Schoch, however, found that the most favourable fractionations were obtained using n-amyl alcohol or "pentasol" (a commercial mixture of various primary amyl alcohols). Schoch also compared the results of various fractionations by the use of polar organic solvents with those obtained by fractionation by leaching of the swollen granules (p. 9-10). Using conditions similar to those of Meyer (26), he has found that the aqueous leaching method has, in most cases, a much lower fractionating efficiency.

As described earlier (p. 13), Kerr and Severson obtained a purified amylose fraction by a combination of the techniques of "aqueous leaching" and "selective precipitation". Their yield of pure material was low, however. Meyer and Rathgeb (48) have recently adopted this combination of techniques.

To effect complete dispersion of the starch, Schoch (p. 10) autoclaved it with butanol at 120°C for several hours. This heat treatment was criticised on the grounds that it was likely to occasion some kind of hydrolytic degradation. Hopkins and Jelinek (49) and Higginbotham and Morrison (47) have found that the use of autoclaving to effect dispersion of the starch is both unnecessary and unsuitable. To avoid this treatment, Haworth, Sagrott and Peat (32) developed a method whereby the starch was given the minimum possible time of heating during dispersion (in some cases only 20 minutes stirring at 100°C and at atmospheric pressure being found necessary). It was

then found possible to add the precipitant, thymol, to the solution which had been cooled to 30°C. The amylose complex deposited after several hours standing at this temperature.

Higginbotham and Morrison (47) have investigated the precipitation method in great detail, using pyridine and butanol as the precipitating agents. They have found that the reprecipitations of the amylose fraction must be carried out in very dilute solution (0.2%). After several reprecipitations (usually five), they obtained amylose fractions (which represented over 80%, sometimes over 90%, of the total amylose in the starch) from potato, sago, tapioca and maize starches which adsorbed very high proportions of their own weight of iodine (see p. 13). After reprecipitation of these amylose fractions from even more dilute solution (0.1%), the iodine affinity was not increased.

The efficiencies of many methods of fractionation have not yet been compared with each other. The comparison has been rendered more complicated by the recent discoveries that the amylose and amylopectin components can be fractionated themselves. Kerr (50,51), Meyer (52) and Schoch (53) have all developed methods capable of subfractionating the amylose and amylopectin components.

THE CHEMISTRY OF AMYLOSE.

Although Maquenne in 1905 had indicated several of the properties of amylose (p. 7), the chemistry of this fraction remained inexact until after the properties of the "synthetic starch" of Hanes (p. 8) had been studied.

Hanes prepared the substance by the action of the enzyme; potato phosphorylase (or P enzyme) on glucose-1-phosphate. This enzyme converted the glucose into a polysaccharide, a large proportion of which separated from solution in a granular form. The properties of this granular substance, for instance, its optical rotation in solution, were, in many cases, similar to those of natural starch. But in other properties it differed from ordinary starch. It gave a more intense blue coloration with iodine; it was less stable in aqueous solution; and, most important, it was completely hydrolysed to maltose by the action of β-amylase. After methylation, too, differences were noted. Haworth, Heath and Peat (54) showed that methanolysis of the methylated "synthetic starch" yielded not more than 1.5% of 2:3:4:6-tetramethyl methyl-D-glucoside. This indicated that one non-reducing terminal group was present per 80-100 glucose residues. Estimations of the reducing power of the "synthetic starch" indicated a molecular size of about 100 units. This figure was also indicated by viscosity measurements on solutions of the methylated "synthetic starch". From these results it was concluded that the "synthetic starch" of Hanes was an unbranched polymer of D-glucopyranose residues joined by 1:4-α-glucosidic linkages. Hassid, Cori and McCready (55) have prepared a "synthetic starch" from glucose-1-phosphate, using muscle phosphorylase. In this case, it was found that the yield of 2:3:4:6-tetramethyl methyl-D-glucoside resulting from the methanolysis of the methylated derivative was of the order of 0.5% or even less, indicating the presence of one non-

reducing terminal group per 200-250 glucose residues.

It was soon found, by comparison of the properties of the "synthetic starch" with the amylose derived from starch, that many similarities existed. In fact, the "synthetic starches" could be described more exactly as "synthetic amyloses".

Natural amylose was found to give a very intense blue colour with iodine; to retrograde from aqueous solution on standing; and to be quantitatively converted to maltose by β-amylase, provided that sufficient care was taken to prevent the amylose retrograding into an insoluble form which was immune to further enzyme action. The results of the methanolysis of the methylated amylose fractions were also similar. Very small quantities of tetramethyl methyl-D-glucoside were isolated. The results obtained by several workers are given in Table III.

TABLE III.

Author	Ref.	Source of Amylose	% of tetramethyl methyl D-glucoside found	Calculated No. of glucose residues per non-reducing end-group.
Meyer, Wertheim and Bernfeld	(56) (57)	Maize	0.32	200-300
		Potato	0.40	200-300
Hess and Krajnc	(58)	Potato	0.40	200-300
Hassid and McCready	(59)	Potato	0.32	200-300
Brown, Halsall, Hirst and Jones	(41)	Sago	see p. 25	250±100
	(41)	Potato	* see below	200±100
Bourne, Fantes and Peat	(60)	Potato(1)	see p. 25	191± 10
	(60)	Potato(2)	see p. 25	187± 10

* Results for this sample of potato amylose were calculated from those obtained for a fraction which contained 20% amylopectin as impurity, and for which allowance was made.

These results indicated the presence of one non-reducing terminal group per 150-300 non-terminal glucose residues. The

value of 150-300 cannot be accepted, however, without question. It represents, perhaps, the minimum value for the amylose fraction since the presence of a trace of amylopectin, whose methylated derivative yields 4-5% of "end-group" would affect the yield of 2:3:4:6 tetramethyl methyl-D-glucoside appreciably.

Meyer, Wertheim and Bernfeld, and Hess and Krajnc carried out their work before the development of the quantitative method of Bates, French and Rundle (p. 12). It is not, perhaps, correct to compare all the results in Table III on a strictly equal basis. The work of Higginbotham (p. 13) has indicated that pure amylose takes up 21.5% of its own weight of iodine. Doubt must, therefore, be cast on results from those amylose samples, the iodine uptake of which does not reach this figure. Thus an amylose sample which takes up only 20% of its own weight of iodine can only be 93% pure. The sample of sago amylose studied by Brown, Halsall, Hirst and Jones (Table III) was shown, however, to be 100% pure.

In spite of the possibility that some of the methylated amyloses (Table III) contained traces of amylopectin, there is evidence from other sources that the figure of one non-reducing terminal group per 150-300 glucose residues is of the correct order.

Brown, Halsall, Hirst and Jones (41) have determined the proportion of such terminal groups present in amylose by measuring the amount of formic acid liberated on oxidation of the polysaccharide, using potassium periodate (see p. 41-43). They found that a sample of potato amylose (which contained 10%

amylopectin, but for which allowance was made) contained one non-reducing terminal group per 500 glucose residues.

Potter and Hassid (61,62) have carried out "end-group" determinations of amylose by periodate oxidation. They have also determined the average number of glucose units per amylose molecule (63). This was obtained by calculation from the number-average molecular weight which was determined by osmotic pressure measurements on the amylose acetate in chloroform solution. The results are shown in Table IV.

TABLE IV.

Source of Amylose	Reference	No. of glucose residues per non-reducing terminal group by periodate oxidation.	Average number of glucose units per mol. from osmotic pressure measurements.
Tapioca	61,63	980	1,300
Potato	"	980	930
Wheat	"	540	860
Corn	"	490	800
Sago	"	420	740
Easter lily	"	640	620
Apple	62	530	560

Other workers (64,65) have determined the number-average molecular weights of amylose derivatives by osmotic pressure measurements. Their results are summarised in Table V.

TABLE V.

Author	Ref.	Source of Amylose	Derivative formed	Solvent used	No. of glucose residues per molecule
Meyer, Bernfeld and Hohenemser	64	Corn	Acetate	Tetrachloroethane	270
Foster and Hixon	65	Corn	"	Chloroform	260
" " "	65	Tapioca	"	Chloroform	460

Meyer, Noelting and Bernfeld (66) have recently developed

a method for determining the degree of polymerisation of naturally occurring polysaccharides. The method depends upon the interaction of the reducing group of the carbohydrate with 3:5-dinitrosalicylic acid in alkaline solution. It is believed that the 3:5-dinitrosalicylic acid is quantitatively reduced to amino-nitrosalicylic acid. The latter product is coloured, and its concentration is measured photometrically. A standardisation curve is prepared, using maltose. Thus the degree of polymerisation of a polysaccharide is given by the relationship.

$$\text{Degree of Polymerisation} = \frac{2 \times \text{Wt. of Polysaccharide}}{\text{Wt. of Maltose}} \text{ for two}$$

solutions having the same colorimetric absorption, i.e., the same reducing power. Meyer has compared results obtained by this method with results from other sources, and found excellent agreement.

From these results, it is readily concluded that amylose is a long chain compound. There is further evidence of a different nature to confirm this. X-ray diffraction patterns obtained by Rundle and co-workers (67,68) with the blue coloured iodine-complex indicate that amylose contains long chains which are arranged in the form of helices, each turn of the helix being made up of about six glucopyranose units. The results also indicate that the iodine atoms are located inside the helix. Such an arrangement is possible with α-linkages. Freudenberg (69) had earlier used the idea of the helical structure to explain the characteristic blue colour. The hydrogen atoms at C_1 and C_4 on either side of the glucosidic bridge (see

fig. II, p. 3) are present to form a hydrocarbon lâning. Since iodine gives blue coloured solutions in hydrocarbons, this was held by Freudenberg to explain the phenomenon.

The observations of Rundle and Baldwin (70) that the amylose-iodine complex shows dichroism of flow, the absorption of light being strongest along the flow lines and least at right angles to them, also indicate that the iodine molecules are arranged with their axes parallel to the long axis of the amylose-iodine complex.

X-ray fibre patterns can also be obtained from amylose and its derivatives (71) when these are in the form of fibres. These fibres have properties similar to the fibres of cellulose and its derivatives. Cellulose is well known to exist in the form of a long chain of glucose units.

These results, however, do not prove whether amylose consists of unbranched or very slightly branched chains. Several workers (39,72,73) have suggested that various amyloses possess different slight degrees of branching due to differences in physical properties. A search of the hydrolysis products of a fully methylated amylose might throw light on this problem. The quantity of dimethyl glucose which might be expected, if slight branching did occur, would be very small indeed. Earlier workers had no satisfactory method of separating and identifying very small quantities.

The development of partition chromatography on paper by Consden, Gordon and Martin (74) in 1944, has produced the desired method. These workers separated and identified the

amino-acids obtained from the hydrolysis of proteins. Only very small quantities of material (less than 1 mgm.) were required, and substances present in very small concentration could be identified. Partridge (75) showed that mixtures of sugars could be separated and identified in a similar manner on this micro scale. Hirst, Jones and co-workers (76,77,78) developed quantitative methods for the estimation of the different sugars, including methylated sugars, separated by this method. Besides achieving success on the micro scale, Jones and co-workers (79) developed methods for the separation of mixtures of sugars on a larger scale by the use of a column of powdered cellulose, and an automatic device for collecting the column eluate, which could readily be fractionated. Thus by utilisation of these methods, it now appears possible to solve the problem whether amylose is slightly branched or not.

The possibility exists, however, that the 2:3:6-trimethyl glucose produced on hydrolysis of the methylated derivative may be demethylated, to some extent, to dimethyl glucoses during the experiment. Bell (80) has shown recently that the extent of demethylation of 2:3:6-trimethyl glucose is small generally. By the use of the chromatographic methods outlined above, it will be possible to examine thoroughly the extent of demethylation and make any necessary allowances for it, in studying the dimethyl glucoses from the methylated amylose.

The proportion of 2:3:4:6-tetramethyl methyl-D-glucoside obtained from the hydrolysis of methylated amyloses has been shown to be about 0.4% (Table III, p. 19). The estimation of

such a quantity is not an easy matter. Earlier workers (54-57) obtained this component by fractional distillation in vacuo, and estimated it, in a mixture with some 2:3:6-trimethyl methyl-D-glucoside, by refractive index measurements. Fractional distillation, however, has disadvantages in that pyrolysis, demethylation and non-quantitative recovery of the sugars are unavoidable. In addition, relatively large quantities of material are required. Bell (81) and Jones (82) have developed partition chromatographic methods, using columns of silica gel and alumina respectively, suitable for the separation of the "end-group" from the hydrolysis mixtures of methylated sugars.

Bourne, Fantes and Peat (60) have used a modification of Bell's method to determine the proportion of "end-group" in methylated potato amylose (Table III, p. 19). Brown, Halsall, Hirst and Jones (41) used the alumina column method to determine the proportion of "end-group" in methylated sago amylose. The method of separation on a cellulose column, however, presents the third, and perhaps the most suitable method of separating, quantitatively, the very small proportions of "end-group" fraction.

SUMMARY: From the evidence available at present, amylose appears to consist of long chains of 1:4-α-linked glucopyranose units. An individual amylose is probably a mixture of polymeric homologues consisting of long chains of several hundred units. It is not yet known whether the chains are very slightly branched or not. The amyloses of different starches appear to be similar in reaction but are not identical.

Although success has been achieved in purifying the amylose fraction, no suitable method for purifying amylopectin has been discovered. The non-precipitated amylopectin fractions obtained by the various precipitation methods (p. 11) have been found to contain some 2-8% of amylose (29,40) as determined by potentiometric titration. All attempts to remove this amylose have failed so far. It is not impossible that this small quantity of amylose is linked to the amylopectin by primary valencies as the difficulties in fractionation would seem to indicate (39).

The conditions of the initial precipitation of the amylose complex must be chosen to keep the amount of amylose in the non-precipitated fraction as small as possible. Higginbotham and Morrison (47) in their fractionation studies (p. 17) have isolated only one sample of amylopectin which has contained no amylose as impurity. This was obtained by the dispersion of maize starch in water containing 20% (v/v) pyridine, followed by saturation with n-butanol. The yield of non-precipitated fraction was low, however.

CHEMISTRY OF AMYLOPECTIN.

Since the amylose content has been estimated to be 15-25% in the commonly occurring starches, amylopectin is, therefore, the major component of these varieties. Earlier studies on the chemistry of whole starch were made using these starches, principally potato and wheat starches. Subsequent studies on the amylopectins of these starches have shown that conclusions reached on the structure of the whole starches have applied essentially to its major component. This indicated that

amylopectin has a much more complicated structure than amylose.

Estimations of the apparent "chain length" of various starches was shown to be 24-30 units (Table I, p.6). Since the proportion of "end-group" in the amylose fraction has been shown to be about 0.4% (p. 19), it can be calculated that the apparent "chain length" of the amylopectin component is 20-25 units. Meyer, Wertheim and Bernfeld (56,57) found that methylated potato and maize amylopectin both yielded about 4% of 2:3:4:6-tetramethyl methyl-D-glucoside which meant that one glucose residue in 25 was a non-reducing terminal group. Hess and Krajnc (58) obtained the same result with another sample of potato amylopectin.

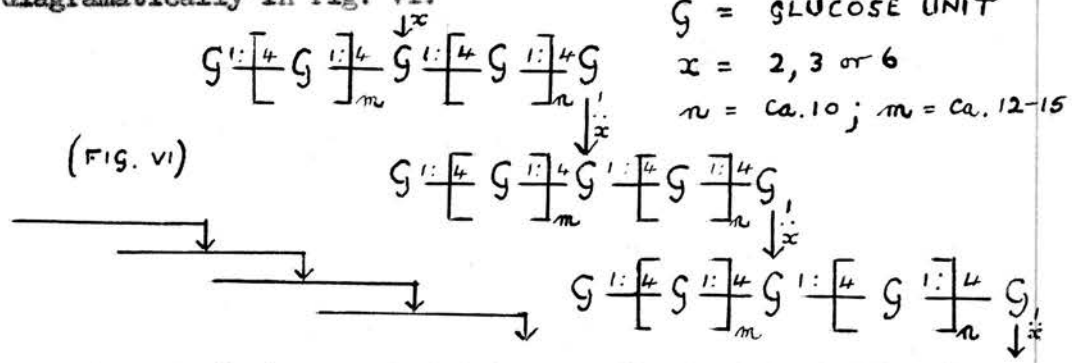
The dimethyl glucoses present in the hydrolysis products of methylated whole starch, in so far as they are not the result of demethylation of trimethyl glucose (see p. 24), must have originated essentially from the methylated amylopectin since the methylated amylose, even is slightly branched, would yield little.

Since amylose has been shown to be completely converted to maltose by the action of β-amylase, it follows that the limit dextrin, resulting from the action of the enzyme on whole starch, is a product of the amylopectin fraction. Thus allowing for the conversion of the amylose present in the starch, it can be calculated that between 50 and 55% of the amylopectin should be converted to maltose. Meyer and Bernfeld (83) have found that this is the case with potato and maize amylopectins.

Similarly the high molecular weight of starch may be

attributed to the amylopectin fraction.

The first satisfactory structure for starch, and therefore for amylopectin, was put forward by Haworth, Hirst and Isherwood (84) in 1937. This structure adequately explained most of the known reactions of starch. Haworth, Hirst and Isherwood suggested that starch consisted of a number of "repeating units", each unit being a straight chain of about 24 glucose units joined by 1:4-α-linkages. The "repeating units" were joined together by primary valencies to form a large molecule with a high molecular weight. The mode of union is shown diagramatically in fig. VI.



Haworth (85) described this as a "laminated structure", although it should be realised that a series of chains in three dimensions, and not a series of parallel plates, is being dealt with.

This structure explained why starch was almost non-reducing and why almost equal quantities of dimethyl glucose and tetramethyl glucose were found on hydrolysis of the methylated starch. The reducing end of each repeating unit was joined glucosidically, in some manner, to one of the available hydroxyl groups of one of the glucose residues in the next chain. It did not prove easy, however, to determine the exact nature of this

"inter-unit" bond.

It had been recognised earlier (86) that starch and its derivatives could be degraded by relatively mild acid treatment yielding products of a lower molecular weight. This indicated that there were, in starch, certain linkages which were weaker than the 1:4-α-glucosidic linkages. These weaker linkages were deemed to form, in part, the "inter-unit" bonds. Hirst and Young (19) reasoned that it might be possible to break the weaker linkages and convert the methylated starch into a product which was the ether of a single "repeating unit". Their experiments yielded important results.

Fully methylated rice starch was heated with a solution of oxalic acid in aqueous methanol, and the process was interrupted at intervals. A series of fractions was obtained and purified. A homogeneous product of molecular weight of about 20,000 (determined by several physical methods) was obtained, which was stable to further attack by the acid solution. During the reaction or "disaggregation" as it was called, the optical rotation remained almost constant and there was no indication of the formation of reducing sugars. The final "disaggregated" product was methylated to substitute any hydroxyl groups which had become exposed during the process and then hydrolysed. It was found that the percentage of "end-group" was almost the same as in the original methylated rice starch, indicating that few, if any, of the 1:4-α-linkages had been broken.

The results indicated that a portion of the "inter-unit" bonds was broken. The derivative, which was finally obtained,

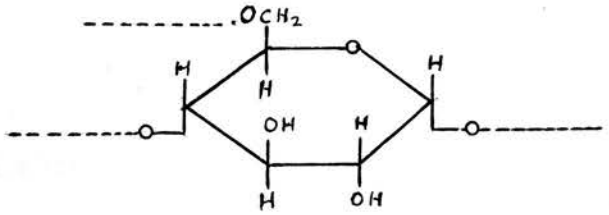
was considered to be composed of four (or probably five) "repeating units". Although it is difficult to explain the exact significance of this molecular size, the important point remains that the average chain length of the "repeating unit" stays constant during the "disaggregation" process, whilst the molecular weight is reduced.

Bawn, Hirst and Young (87) studied the kinetics of the "disaggregation" reaction. By plotting the molecular weights of the fractions against the time taken for "disaggregation", the rate of the process could be followed. It was calculated to be six or seven times slower than the rate of hydrolysis of methylated inulin, and to have an activation energy of 25,000 cals.. Since the physical molecular weight of methylated inulin agrees with the value found by "end-group" assay, it follows that there is no possibility of aggregation. Hydrolysis must, therefore, involve the breaking of glucosidic links.

The next immediate problem to be settled was to what actual point of a glucose unit of one chain the reducing end of the next chain was linked. Barker, Hirst and Young (88) studied the hydrolysis products of the final "disaggregated" fraction (p. 29). They found that the proportion of dimethyl sugar in this fraction was 1.9%. (The initial product contained 3.3% of dimethyl sugar. This decrease in the proportion of dimethyl sugar is in agreement with the theory that the "disaggregated" product should contain fewer branching points.) Examination of this dimethyl fraction showed that it was principally 2:3-dimethyl D-glucose. This indicated that a large proportion of

the "inter-unit" bonds were 1:6-linkages (fig. VII).

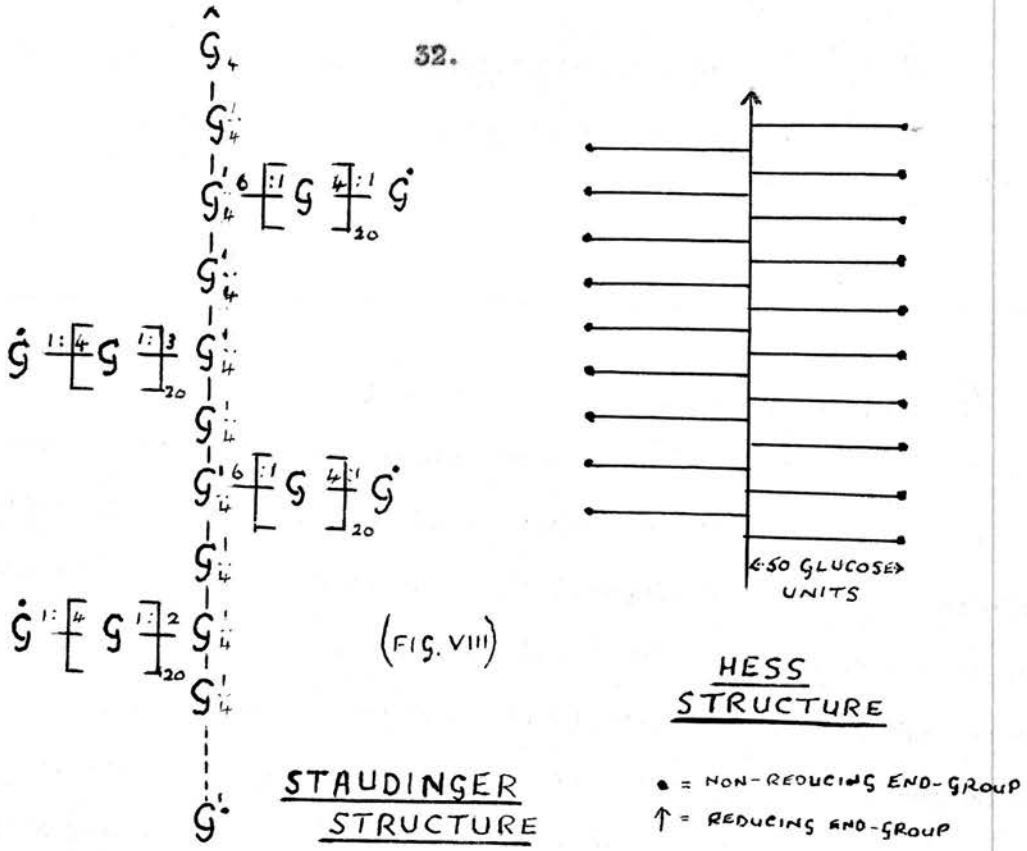
(FIG. VII)



The yield of 2:3-dimethyl D-glucose, however, did not account for all the branching points, and there was also the possibility that part of it had arisen by demethylation of 2:3:6-trimethyl D-glucose. Thus it could only be concluded that some, but not all, of the "inter-unit" bonds were 1:6-linkages. There was no evidence to prove that the bonds which were broken during the "disaggregation" process (these represented about one-fifth of the total "inter-unit" bonds) were 1:6-linkages.

Freudenberg and Boppel (89) carried out investigations on the nature of the "inter-unit" bond, concurrently with Hirst and Young. These workers subjected starch to repeated methylation with sodium and methyl iodide in liquid ammonia and obtained substances which were completely substituted (i.e., with a theoretical methoxyl value of 45.6%). 2:3-dimethyl D-glucose was also found to be the principal constituent of the dimethyl fraction from the hydrolysates of these fully methylated starches.

Other structures for starch (fig. VIII) were proposed by Staudinger (90) and by Hess and Lung (91)



(FIG. VIII)

Both of these postulated the presence of a main chain of glucose residues to which were attached side-chains terminating in non-reducing groups. These structures, however, did not explain the results obtained by Hirst and Young's "disaggregation" experiments. The effect of "disaggregation" on such structures would be likely to produce low molecular weight products or certainly final products which were not homogeneous. Neither was found to be the case.

These structures were also criticised by other workers (83,92) on the basis of studies on the breakdown of amylopectin by hydrolytic enzymes.

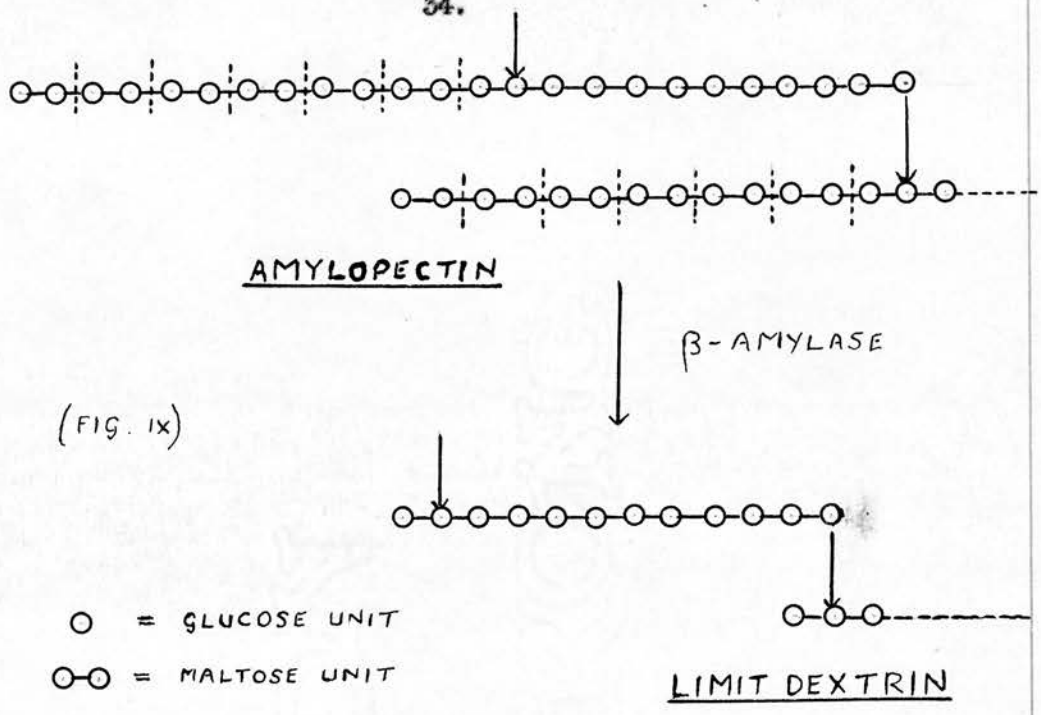
When amylopectin is treated with β-amylase, maltose (50-55%) and a limit dextrin (45-50%) are produced (83). The dextrin is stable to further attack by β-amylase unless it is treated with acid or with other enzymes (83,92,93).

Investigations on this limit dextrin by various workers (94) showed that it possessed one non-reducing terminal group per 11-12 glucose residues and that it had a high molecular weight. It appeared to be a genuine part of the amylopectin structure.

It had been recognised (95) that the β-amylase attacked initially at the non-reducing end of the glucose chain, removing maltose units successively, one at a time. The formation of a dextrin, stable to further attack by β-amylase, indicated the presence of an "obstruction" in the glucose chain.

These facts provided evidence to support the "laminated" structure. They tended to invalidate the structures of Staudinger and of Hess and Lung (fig. VIII) since these required the formation of higher yields of maltose and of limit dextrins of lower molecular weight than were actually obtained.

Since 55% of maltose was formed by β-amylase action, this would mean that the "unit chains" of the "laminated" structure were shortened by just over half their length. The presence of the "obstruction" almost certainly indicated that a point had been reached to which another "repeating unit" was attached (see fig. IX). This, therefore, supplied indirect evidence on a question about which purely chemical evidence had, so far, provided no answer, i.e., to which point along the "unit chain" the next chain was joined. It could now be said that the branching point appeared slightly more than half way along the chain from the non-reducing end-group.



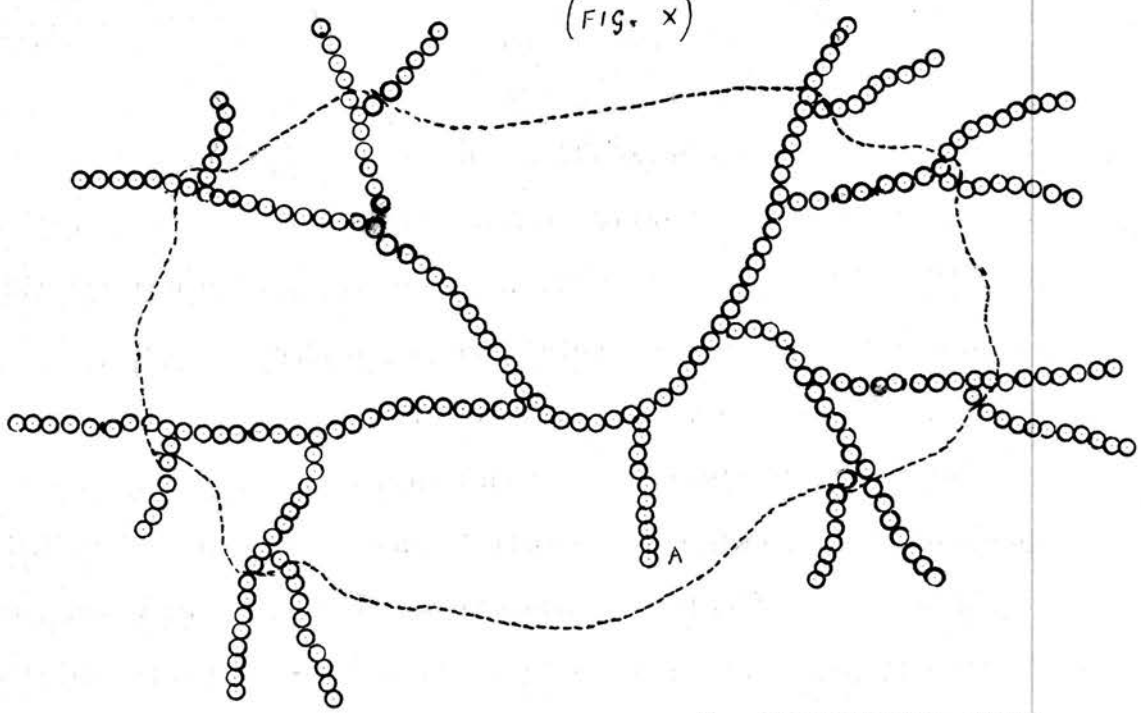
(FIG. IX)

The average chain length of the limit dextrin should be 11-12 units, since that of the original amylopectin was about 25 units. This was shown to be the case (p. 33).

Meyer and Bernfeld (83) criticised the "laminated" structure and suggested an alternative structure (fig. X) based on multiple branching. It will be recognised that Meyer's structure is similar in many ways to the "laminated" structure. If a comparison, however, is made of the structures of the limit dextrins obtained after the action of β-amylase on amylopectin, it will be observed that the "laminated" structure and the "Meyer" structure show different interpretations. In the "laminated" structure (fig. IX), the dextrin is found to consist of almost a straight chain of glucose units, whereas it still appears as a highly ramified structure on Meyer's interpretation (i.e., that structure enclosed by the dotted lines (fig. X)). Now the

acetylated derivative of a dextrin of ramified structure should still show the same properties of the original acetylated amylopectin forming brittle films which are easily powdered, whereas the acetylated derivative of an almost straight chain dextrin should show the properties of an acetylated amylose forming fibrous films which cannot be powdered. Since the actual acetylated dextrin was a brittle solid resembling acetylated amylopectin, Meyer concluded that his own structure was the correct one.

(FIG. X)



A = REDUCING END-GROUP

O = GLUCOSE UNIT

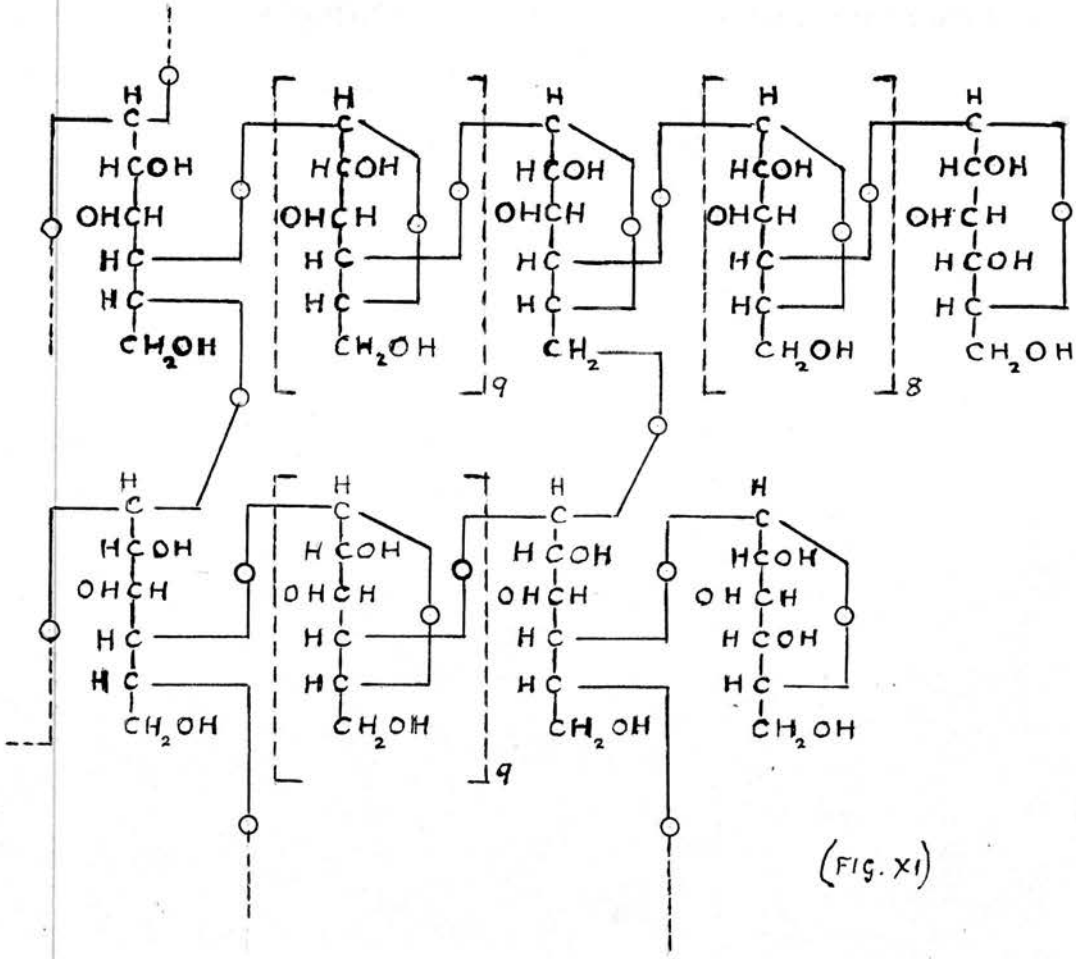
(The structure inside the dotted line is the limit dextrin as postulated by Meyer).

However, this evidence is very slight. In addition, the results of the "disaggregation" experiments (p. 29) are not readily explained on the "ramified" structure, since if one in five of the "inter-unit" bonds are broken then there is no reason

to assume that the product obtained would be homogeneous; rather, one would expect it to be somewhat heterogeneous. At the moment, therefore, it is perhaps more reasonable to accept the simpler "laminated" structure on the present evidence.

Another structure for amylopectin has recently been proposed by Pacsu (96) who suggested that, in addition to the normal 1:6-linkages between "repeating" units, there are acetal linkages (fig. XI) which are more labile to hydrolysis and would be broken under conditions similar to those used by Hirst and Young (p. 29) during their "disaggregation" experiments. It is not, at the moment, possible to estimate whether such acetal bonds are present in starch or not.

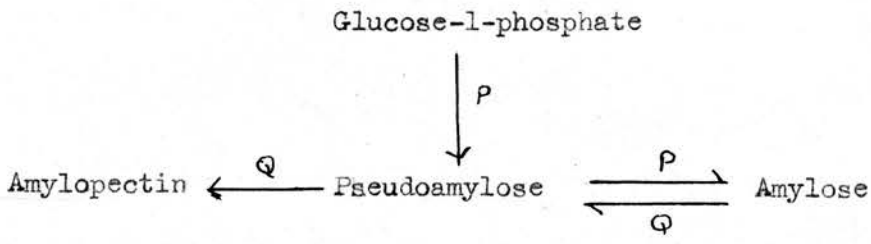
Pacsu (96) also postulated the presence of hemi-acetal bonds in starch. The work of Halsall, Hirst and Jones (p. 41) has shown that it is very unlikely that this type of linkage exists in starch.



(FIG. XI)

Since the discovery by Hanes (pp. 8,17) that amylose could be synthesised by enzymes, research has been carried out to find the conditions under which amylopectin could be built up. Haworth, Peat and Bourne (97) in 1943 isolated an enzyme from potato juice which they named Q enzyme, capable of acting on amylose to yield another polysaccharide which produced a red coloration with iodine. The Q enzyme, itself, did not show any synthetic activity with glucose-1-phosphate unless the potato phosphorylase (or P enzyme) was present. With both enzymes present, glucose-1-phosphate yielded a polysaccharide which was almost certainly amylopectin since it gave a red coloration with iodine; underwent conversion to maltose by β-amylase to the extent of 46%; and possessed one non-reducing terminal group per 20 glucose residues (98).

It was suggested that the glucose-1-phosphate was built into a substance called pseudoamylose, consisting of a chain of 20 glucose units which, in the absence of the Q enzyme, was converted into the long chain amylose of Hanes. The Q enzyme, however, causes the building up of the pseudoamylose into the "laminated" structure of amylopectin. The Q enzyme also breaks amylose down to pseudoamylose. The reactions are formulated in figure XII.



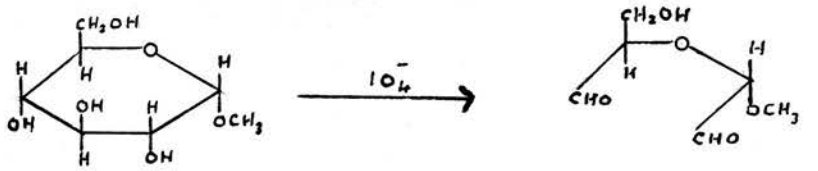
(FIG. XII)

It may prove that this synthetic amylopectin will yield valuable evidence on the structure of the amylopectin molecule.

THE OXIDATION OF STARCH AND ITS COMPONENTS BY THE PERIODATE ION.

Since 1936, the oxidative degradation of α-glycols by periodic acid has found many applications in carbohydrate chemistry. It is only within the last two or three years, however, that satisfactory methods have been developed to apply this oxidative reaction to polysaccharides. Much confirmatory evidence on the structure of amylose and amylopectin has now been gained, mainly through the work of Hirst, Jones and co-workers (41,99,100).

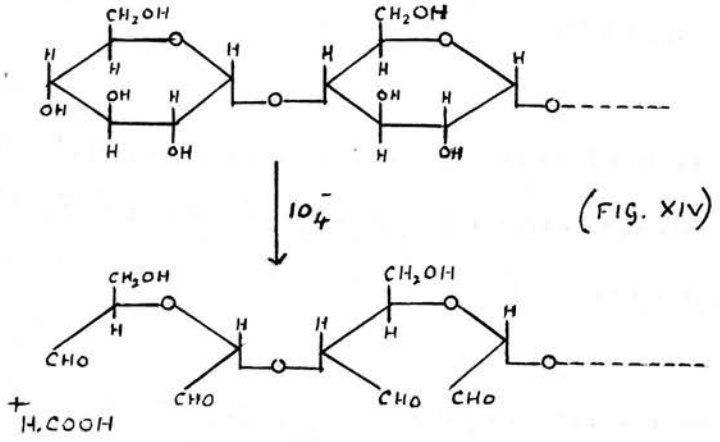
Periodate ions ($-IO_4^-$) selectively oxidise α-glycols, forming dialdehydes (101). When the grouping - CHOH-CHOH-CHOH- is oxidised, a dialdehyde and one molecule of formic acid are produced. Jackson and Hudson (102) utilised this reaction to prove the presence of three contiguous hydroxyl groups, and hence the presence of a pyranose



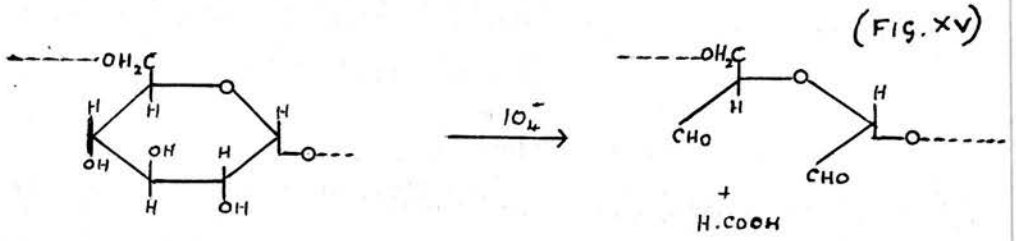
α-METHYLGLUCOSIDE (FIG. XIII)

ring in α- and β-methyl D-glucosides (fig. XIII). They similarly found that all the methylhexopyranosides which they examined yielded one molecule of formic acid on periodate oxidation. Thus it follows that in the case of a polysaccharide composed only of hexopyranose units and which has no appreciable

proportion of reducing end-group, any sugar residue which is linked to another residue only through C_1 (and is, therefore, a non-reducing terminal residue) or through C_1 and C_6 (main chain residue) will give rise to one molecule of formic acid (figs. XIV and XV)



(FIG. XIV)



(FIG. XV)

on periodate oxidation. No formic acid should arise from any other residue. Thus, from the amounts of formic acid produced on periodate oxidation, it appears possible that considerable information about the nature of a polysaccharide molecule would be obtained. It would be necessary, however, to keep a careful control of the conditions as "over-oxidation", i.e., oxidation other than the selective fission of α-glycol groups, is likely to occur. The latter difficulty has generally restricted the usefulness of the periodate method. Hirst, Jones and co-workers (41,99) have developed a method whereby the formic acid

can be satisfactorily estimated and little "over-oxidation" occurs. By this method, the polysaccharide is oxidised in an aqueous solution by a suspension of potassium periodate. As this salt has only a very slight solubility in water, there is insufficient periodate present in solution to permit any significant "over-oxidation". The resulting formic acid is estimated by titration with 0.01N alkali after the addition of ethylene glycol. The latter reduces the excess periodate to neutral iodate and is, itself, oxidised to formaldehyde which is also neutral.

Thus, in the case of polysaccharides built up on the pattern of starch with chains of 1:4:-α-linked hexopyranose residues, the results of the yield of formic acid may be interpreted in the following way. The percentage of glucose residues giving formic acid is first calculated. This is compared with the percentage of non-reducing terminal groups as determined by the methylation technique. If these two results are identical, then it follows that the polysaccharide can contain no residue linked only through C_1 and C_6. If formic acid is produced in excess of that resulting from the non-reducing terminal groups, then it provides a measure of the proportion of sugar residues linked only through C_1 and C_6. If it is found that there are no sugars linked only through C_1 and C_6, then the amount of formic acid produced provides a measure of the percentage of glucose residues present as non-reducing terminal groups.

Brown, Halsall, Hirst and Jones (41) compared the percentage of glucose residues giving formic acid with the percentage of non-reducing end-group as determined by the methylation technique.

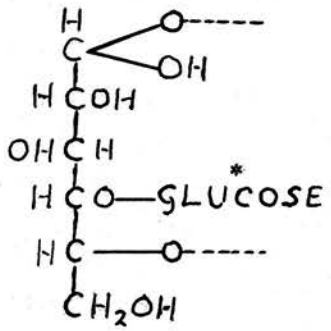
They examined banana, potato, sweet potato and waxy maize starches by both techniques and found that the results were almost the same (Table VI).

This discovery was important from two points of view. Firstly, in the whole starches and thus also in the amylose and amylopectin components, there can be no glucose residues linked only through C_1 and C_6. These workers made the reasonable assumption that, in all starches, there are no glucose residues linked together only through C_1 and C_6. The yield of formic acid can be used, therefore, to determine the percentages of non-reducing terminal groups in all starches.

TABLE VI.

Source of Starch	Amylose Content	Average No. of glucose units per non-reducing end-group (A) By periodate method	(B) By methylation method	Calculated No. of glucose residues per non-reducing end-group in the amylopectin fraction
Banana	21	27	26	21
Potato	18	31	25,28	24,26
Sweet Potato	18	32	28,34	26
Waxy Maize	0-1	20	18	20
Potato Amylopectin	0-1	24	-	24
Acorn (see (14))	20	29	30	24

Secondly, it ruled out the hemiacetal type of linkage proposed by Pacsu and Hiller (96) in one of their formulae for starch. This structure was based on the assumed occurrence of a small proportion of non-cyclic hemiacetal linkages. The essential feature as applied to starch is shown in fig. XVI which depicts a maltose residue in a hemiacetal form, serving as a link between long chains



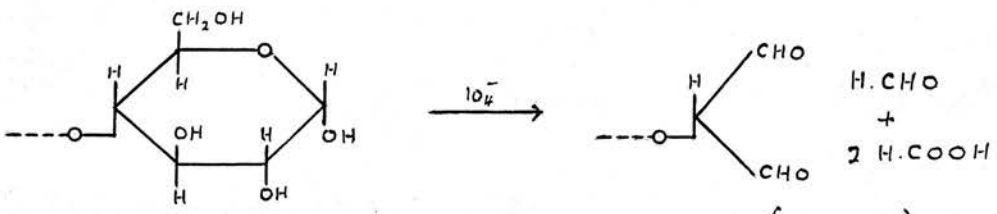
(FIG. XVI)

of 1:4-linked glucopyranose residues. The glucose residue marked * is an end-group, and Pacsu postulates a sufficient number of these cross-linkages to account for the number of end-groups as determined by the methylation method. Inspection of fig. XVI will show that the presence of hemiacetal linkages increases the number of contiguous hydroxyl groups at which oxidative attack by periodate is possible. More than one molecule of formic acid would then be produced per non-reducing terminal group. This, however, does not agree with the experimental results.

The proportion of non-reducing end-group can be determined for the amylopectin fraction in exactly the same way as for the whole starch. It is possible, however, to obtain an indirect result for this fraction in the following way. As a first approximation, the amount of formic acid produced from the long chain amylose component in whole starch can be neglected in comparison with that produced by the amylopectin component. This is feasible if the amylose is present to an extent not exceeding 25%, and provided that the percentage of end-group, reducing as well as non-reducing, in the amylose is less than 1%. In most starches, these conditions are satisfied. The

determination of the amylose content is carried out by the method of Bates, French and Rundle (p. 12). Thus the percentage of non-reducing terminal groups in the amylopectin fraction can be readily calculated. The value found for potato amylopectin (41) agreed with the value calculated from the results for the whole starch (see Table VI).

In the amylopectin fraction, the proportion of reducing end-group is small compared with that of the non-reducing end-group. The amount of formic acid produced from the reducing end-group may be ignored, and calculations based on the assumption that all the formic acid is produced from the non-reducing end-groups. However, in the case of the long chain amylose molecule, the reducing end-group may constitute up to 50% of the total end-group, and formic acid will be produced by both types of end-group. The non-reducing end-group will produce one molecule of formic acid, whereas the reducing end-group will finally give rise to two molecules of formic acid and one of formaldehyde (fig. XVII). Corrections must,



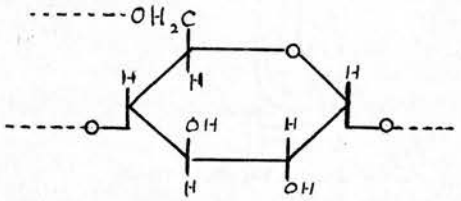
REDUCING END-GROUP

(FIG. XVII)

therefore, be applied before the proportion of non-reducing end-group can be calculated.

The oxidation of amylose and amylopectin by periodic acid illuminates another structural matter. Inspection of fig.XVIII

will show that, when one chain is joined to another through C_6 of a glucose residue, there remains in that residue two contiguous hydroxyl groups



(FIG. XVIII)

at C_2 and C_3. Oxidation with periodic acid will, therefore, disrupt the bond between C_2 and C_3 and, after subsequent hydrolysis, the sugar residue involved in the branching will no longer be recoverable as glucose. On the other hand, if the chain junction is at C_2 or C_3, no α-glycol groupings remain, and there will be no attack by periodic acid. In these circumstances, the glucose residues remain intact and will be recoverable as glucose after hydrolysis. It follows, therefore, that oxidation by periodic acid, followed by hydrolysis and examination of the products, should give information about the mode of "inter-unit" linking in the amylopectin fraction. The presence of small quantities of glucose can be detected and estimated by the paper chromatographic methods outlined on p.23.

Halsall, Hirst, Jones and Roudier (14,100) applied this procedure to potato, acorn, sago and waxy maize starches. In each case, glucose was detected in small amounts. From the results, however, it was concluded that in the amylopectin fraction of these starches, the greater proportion of "inter-unit" linkages (more than 75%) are of the 1:6 variety. It was not found possible to decide whether the remainder were

also 1:6 linkages or were of the 1:2 or 1:3 varieties.

Inspection of the proportion of non-reducing end-group in the various amylopectins (Table VI) shows that they are not similar. It is unlikely that the differences are due to experimental error, especially as the sample of highly purified amylopectin (Table VI) gave almost the same proportion of non-reducing terminal group as calculated from results with the whole starch. All amylopectins appear to have 20-26 glucose residues per non-reducing terminal group. These results indeed suggest that there is a difference in the size of the "repeating unit" of the various amylopectins. Kerr (50) has gone even further and suggested that the amylopectin of an individual starch is composed of a series of homologues with different numbers of "repeating units" joined together.

The most important problem connected with amylopectin, however, remains that concerned with the nature of the "inter-unit" linkages or "branching points". As stated already, the dimethyl glucose and, in particular, the 2:3-dimethyl glucose which is obtained from the hydrolysis products of the methylated whole starch, can be assumed to come from the amylopectin fraction, since the fully methylated amylose is likely to yield little. Whilst these facts indicate the presence of at least some 1:6 linkages, they are by no means conclusive. The complete methylation of starch, without degradation, is seldom achieved. Experimental work has generally been carried out with methylated starches containing only 43-44% O Me, as against the theoretical percentage of 45.6. Thus some of the dimethyl

glucose probably results from incomplete methylation of a glucose residue and not from a fully methylated glucose residue with three other glucose units attached to it.

In spite of this difficulty, however, the development of the new techniques (p. 23) should help to elucidate the nature of the "inter-unit" linkage. Halsall, Hirst, Jones and Roudier (p. 44) have indeed illustrated this by showing that over 75% of the linkages in several amylopectins are of the 1:6 variety. It seems that with the amylopectin fraction, the various structures put forward satisfy most of the known facts, but all the information required to discriminate between them is not yet available.

EXPERIMENTAL.

EXPERIMENTAL.

PREPARATION OF BARLEY STARCH.

Barley ("Pioneer" variety (moisture content - 10.7%) - 1 kilo.) was ground in a "Raymond" laboratory mill, using the screen with $\frac{1}{16}$" apertures. The grist was then extracted with light petroleum (b.r. 40°-60°) in a Soxhlet apparatus for 10 hours to remove fatty substances. After drying at a low temperature to remove solvent, the grist was thoroughly mixed and was extracted for 6 hours by shaking with water (5 litres) in stoppered Winchester bottles.

The suspension was then passed through a 40-mesh sieve to remove larger particles of fibrous matter, and then through a 150-mesh screen to remove finer fibrous particles. The screened suspension was allowed to stand overnight. A deposit of material rich in starch formed at the bottom of the vessel. The supernatant liquid was siphoned off and fresh water was added to attain a specific gravity of 1.030 (by hydrometer). After stirring for 1 hour, the suspension was centrifuged at 1,500 r.p.m. to deposit the starch. Above the starch deposit there was found a well-defined deposit of very fine fibrous material, together with some insoluble protein. This top layer was removed, as completely as possible, by scraping with a spatula and washing with a stream of water. Water was again added to the crude starch to attain a specific gravity of 1.030, and the process was repeated to remove the last traces of fibre.

In order to remove heavy particles, e.g., of sand, a slurry

was made by the addition of water and centrifuged at a low speed (150 r.p.m.) for 30 seconds. The heavy particles were deposited and the starch remained in suspension. This suspension was filtered on a Buchner funnel, using a rapid paper (Whatman No. 41).

The product was dried at 40°C and then ground with mortar and pestle. The fat content was further reduced by the technique of Kerr (103) by refluxing with a methanol-water mixture (85:15 by weight, 200 gm.) After three treatments with this mixture, the product was filtered and washed with water to free from methyl alcohol.

The sample was dried at 40°, reground with mortar and pestle and dressed through a 150-mesh sieve. It was then left exposed in the laboratory to reach equilibrium moisture content before bottling.

Yield of starch, 137 gm.

Examination of the Starch.

The white powder was examined under the microscope. The granules were seen to be of a moderate size (diameter 10-35 μ) and were ovoid to spheroidal in shape. It was observed that very few of the granules were ruptured.

Analysis of the Starch.

Ash (as sulphate), 0.2%.
Moisture Content, 12.2%.
Protein (% Nitrogen x 6.25), 0.3%.
(Nitrogen determined by the micro-Kjeldahl technique).
Optical Rotation (A) in N NaOH solution.

$[\alpha]_D^{18}$, + 157° (C = 1%).

(B) in perchloric acid solution (30%)

$[\alpha]_D^{16}$, + 188° (C = 0.69%).

A dispersion of the starch in water gave a deep blue coloration with iodine.

"Blue Value" (p.15) denoted in the following pages by the letters B.V.), 0.27-0.28.

Hydrolysis with sulphuric acid (A).

Dry starch (30 mgm.) was heated in a sealed tube and hydrolysed at 100°C with 2% sulphuric acid (0.5 ml.) After 7 hours the tube was opened and the acid neutralised with barium carbonate. The barium sulphate was centrifuged off and the resulting clear solution was examined by paper chromatography (76 see p. 23). Glucose only was found to be present.

The experiment was repeated and the glucose was estimated by the method of Flood, Hirst and Jones (76) using ribose as the reference sugar. It was found that the starch gave 97% of the theoretical yield of glucose.

Hydrolysis with Sulphuric Acid (B) and estimation of the glucose with alkaline hypoiodite.

Dry starch (0.1030 gm.) was hydrolysed by boiling with sulphuric acid (2%, 25 ml.) for 7 hours. The acid was neutralised with barium carbonate and the resulting barium sulphate was filtered off and washed. The filtrate was reduced in volume (to 20 ml .) and iodine (0.1N, 20 ml.) and excess of sodium hydroxide solution were then added. A reagent blank was also prepared. The solutions were allowed to stand in the dark for 60 minutes. Sulphuric acid (2N) was then added until the solution was acid and the excess iodine was titrated with standard sodium thiosulphate solution.

Wt. of glucose found = 0.1101 gm.

This constitutes 96.2% of the theoretical yield of glucose from the starch.

Potentiometric determination of Amylose.

The method used was the modification elaborated by Hudson, Schoch and Wilson (40) of the method of Bates, Rundle and French (36). (For method of calculation: see ref. 104).

The amount of iodine (average) taken up by 1 gm. of starch was 0.0402 gm. Higginbotham and Morrison (39) have found that pure amylose takes up 21.5% of its own weight of iodine.

Barley starch, therefore, contains 18.7% amylose.

Acetylation of Barley Starch
By the method of Pacsu and Mullen (105)

Air-dried starch (15 gm.) was dispersed in hot water (500 ml.) and stirred for 1 hour to effect complete dispersion of the granules. The paste was very viscous. Pyridine (1 litre) was added and the pyridine-water azeotrope was distilled off under reduced pressure. To ensure complete removal of the water, a further volume of pyridine (500 ml.) was added and the vacuum distillation continued until the final volume was 500-600 ml. Acetic anhydride (300 ml.) was then slowly added (the containing vessel being surrounded by cold water to prevent any sudden rise in temperature) with stirring. After the addition of the acetic anhydride, the mixture was stirred for 4 hours at room temperature and for 5 hours at 60°. When cool, the solution was poured into ice-cold water. The acetate was washed with water until free from acid, then with alcohol and ether and dried in a vacuum over calcium chloride. Yield, 21.5 gm.

(90% of theoretical).

Found $CH_3 \cdot CO$, 43.3% (theoretical 44.8%).

The acetate was white in colour. It dissolved easily in chloroform, but only with difficulty in acetone.

$[\alpha]_D^{14}$, + 170° (C = 0.5% in chloroform).

Specific viscosity, η_{sp}^{20} = 1.13 (C = 0.4% in meta-cresol). This corresponds to an apparent molecular weight of 458,000 for barley starch itself (see ref. 19).

Deacetylation of the Starch Acetate

By the method of Zémplen and Pacsu (106) using sodium methylate.

Acetylated starch (10 gm.) was dissolved in dry chloroform (250 ml.) in a glass-stoppered bottle (500 ml.) Dry methanol (50 ml.) containing dissolved sodium (50 mgm.) was added slowly, with shaking, to the chloroform solution. The bottle was shaken for 1 day and left standing for 2 more days. A fine white precipitate settled out. This was filtered on a G4 sintered-glass filter and washed with several portions of absolute alcohol until free from sodium, and finally with ether. Yield, 5.5 gm. (95% of theoretical).

Found $CH_3 \cdot CO$, nil.

B.V. = 0.283.

$[\alpha]_D^{18}$ = + 192° (C = 0.6% in perchloric acid (30%)).

FRACTIONATION OF BARLEY STARCH.

A summary of all the results of fractionation experiments is given in Table VII (p. 57). In all cases, the precipitated fraction is referred to as "amylose", and the non-precipitated fraction as "amylopectin", whether or not the fractions contained

the other as impurity.

1A) By the "standard" method of Bourne, Donnison, Haworth and Peat (32) using thymol.

Dry starch (10 gm.) suspended in cold water (40 ml.) was slowly added with continuous stirring to boiling water (300 ml.) The viscosity of the paste was lowered by adding sodium chloride (0.35 gm.) and continuing to stir the boiling solution for 20 minutes. The paste was cooled to 70° and centrifuged (at 10,000 r.p.m.) for 10 minutes. A residue of undispersed material was obtained.

The temperature of the paste was adjusted to 30°C and powdered thymol (0.67 gm.) added with stirring. The mixture was left at 30° for 60 hours, during which time the insoluble amylose-thymol complex settled out. This was removed in a centrifuge (2,000 r.p.m.), washed by being stirred with water saturated with thymol (100 ml.), and again separated. The washing process was repeated with 2 fresh portions of water saturated with thymol. The precipitate was dehydrated and freed from thymol by being repeatedly triturated with alcohol and then ether. It was dried in a vacuum over phosphorus pentoxide.

The amylopectin fraction was precipitated by the addition of alcohol (450 ml.) to the concentrated mother liquor (150 ml.) after the removal of the amylose complex. It was washed with alcohol and ether, and dried over phosphorus pentoxide.

Found	"Amylose"	"Amylopectin"	Residue
Yield	1.04 gm.	6.8 gm.	2.1 gm.
B.V.	0.80	0.150	0.45

	"Amylose"	"Amylopectin"	Residue
Uptake of iodine det. by potentiometric titration	12.80%	1.73%	-
% Amylose in Fraction	60	8	-

1B) Using thymol.

Method 1A was applied, but deacetylated starch (5 gm. - see p. 51) was used.

Found	"Amylose"	"Amylopectin"	Residue
Yield	0.9 gm.	4.0 gm.	0.1 gm.
B.V.	0.91	0.141	-

1C) Using thymol.

Method 1A was applied with one modification. After cooling to 70° (see p. 52) the paste was first subjected to 5 minutes high-speed stirring in an "Ato-Mix" disperser. The temperature was adjusted to 70° and the paste was centrifuged as before. (5 gm. starch used).

Found	"Amylose"	"Amylopectin"	Residue
Yield	0.7 gm.	4.1 gm.	0.2 gm.
B.V.	1.03	0.141	-

1D) Using thymol.

Method 1A was applied, again with one modification. The boiling solution was stirred for 3 hours instead of 20 minutes. (5 gm. of starch used).

Found	"Amylose"	"Amylopectin"	Residue
Yield	0.9 gm.	3.9 gm.	0.2 gm.
B.V.	1.03	0.128	-

2) By the method of Higginbotham and Morrison (47) using butanol.

Dry starch (5 gm.) suspended in cold water (20 ml.) was slowly added with continuous stirring to water (200 ml.)

saturated with n-butanol (40 ml.) at 90°. The stirring was continued at this temperature for 2½ hours. The paste was cooled to 70°C and centrifuged (at 10,000 r.p.m.) for 5 minutes. (It was found that, if the centrifuging was continued for more than 5 minutes, the paste cooled to a temperature below 50°, and precipitation of the amylose-butanol complex took place).

The temperature of the solution was adjusted to 90°. The solution was then transferred to a Dewar flask and allowed to cool very slowly. After 60 hours, the amylose-butanol complex was removed in a centrifuge (5,000 r.p.m.) and washed with several portions of water saturated with butanol. The precipitate was dehydrated and freed from butanol by being repeatedly triturated with alcohol and then ether. It was dried in a vacuum over phosphorus pentoxide.

The amylopectin fraction was precipitated by the addition of alcohol (450 ml.) to the concentrated mother liquor (150 ml.) after the removal of the amylose complex. It was washed with alcohol and ether and dried over phosphorus pentoxide.

Found	"Amylose"	"Amylopectin"	Residue
Yield	1.0 gm.	3.8 gm.	0.2 gm.
B.V.	0.90	0.137	-

3) By the method of Higginbotham and Morrison (47) using pyridine.

Dry starch (5 gm.) suspended in water (17.5 ml.) was added with stirring to water (200 ml.) at 90°, containing pyridine (37.5 ml., redistilled over potassium hydroxide). The procedure of Method 2 was then followed exactly. The amylose-pyridine complex was also washed with butanol saturated with

water.

Found	"Amylose"	"Amylopectin"	Residue
Yield	1.0 gm.	3.8 gm.	0.2 gm.
B.V.	0.99	0.111	-
Uptake of iodine det. by potentiometric titration	15.28%	-	-
% Amylose in Fraction	71	-	-

4A) By the method of Higginbotham and Morrison (47) using pyridine and butanol.

Dry starch (5 gm.) suspended in water (20 ml.) was added with stirring to water (180 ml.) at 90°, containing pyridine (50 ml.) The paste was stirred for ½ hour and then subjected to 5 minutes high-speed stirring in an "Ato-Mix" disperser. The paste was cooled to 70° and centrifuged (at 10,000 r.p.m.) for 5 minutes. The temperature of the paste was readjusted to 90°C, and sufficient butanol was added to saturate the solution. After transferring to a Dewar flask, the solution was allowed to cool very slowly. The procedure of Method 2 was then followed to isolate the individual fractions.

Found	"Amylose"	"Amylopectin"	Residue
Yield	2.5 gm.	2.2 gm.	0.3 gm.
B.V.	0.41	0.087	-
Uptake of iodine det. by potentiometric titration	-	0.61%	-
% Amylose in Fraction	-	2.8	-

4B) Using pyridine and butanol.

Method 4A was applied with one modification. The paste was stirred for 1 hour instead of ½ hour.

Found	"Amylose"	"Amylopectin"	Residue
Yield	2.7 gm.	2.1 gm.	0.2 gm.
B.V.	0.42	0.067	-
Uptake of iodine det. by potentiometric titration	-	0.43%	-
% Amylose	-	2	-

4C) Using pyridine and butanol.

Method 4A was applied, again with one modification. The paste was heated, for 3 hours instead of ½ hour.

Found	"Amylose"	"Amylopectin"	Residue
Yield	2.8 gm.	2.1 gm.	0.1 gm.
B.V.	0.43	0.067	-

The results of all the fractionation experiments are summarised in Table VII.

TABLE VII

CONDITIONS OF DISPERSION					METHOD	PRECIPITANT	AMYLOSE FRACTION		APPROX. PERCENTAGE OF TOTAL AMYLOSE IN PRECIPITATED FRACTION.	AMYLOPECTIN FRACTION.		APPROX. PERCENTAGE OF TOTAL AMYLOPECTIN IN NON-PRECIPITATED FRACTION.
CONC. OF STARCH (%)	TEMP. (°C)	TIME (HRS.)	SOLVENT	STARCH DISSOLVED (%)			B.V.	% AMYLOSE		B.V.	% AMYLOSE	
3	100	1/3	WATER CONT. NaCl.	58	1A	THYMOL	0·80	60⊗	32	0·150	8·0 ⊗	75
3	100	1/3	"	98	1B	"	0·91	66	63	0·141	7·4	89
3	100	1/3 *	"	96	1C	"	1·03	74	56	0·141	7·4	94
3	100	3	"	96	1D	"	1·03	74	72	0·128	6·6	92
2	90	2½	WATER SAT. WITH BUTANOL	96	2	BUTANOL	0·90	65	68	0·137	7·1	87
2	90	2½	WATER CONT. PYRIDINE	96	3	PYRIDINE	0·99	71⊗	76	0·111	5·3	89
2	90	½ *	WATER CONT. PYRIDINE AND BUTANOL.	94	4A	PYRIDINE & BUTANOL	0·41	29	85	0·087	2·8 ⊗	52
2	90	1 *	"	96	4B	"	0·42	30	89	0·067	2·0 ⊗	51
2	90	3 *	"	98	4C	"	0·43	31	91	0·067	2·0	50

* DENOTES THAT 5 MINUTES HIGH SPEED DISPERSION WAS GIVEN

⊗ DENOTES THAT AMYLOSE CONTENT WAS DETERMINED BY POTENTIOMETRIC TITRATION. ALL OTHER AMYLOSE CONTENTS WERE CALCULATED FROM THE 'BLUE VALUES'.

Reprecipitation of an "amylose" fraction using thymol.

Dry, powdered amylose (1.5 gm.; B.V., 1.03, prepared by Methods 1C and 1D (p. 53) was added slowly with stirring to boiling water (100 ml.) The procedure of Method 1A (p. 52) was then followed, the paste being stirred for 20 minutes as before.

Found	"Amylose"	"Amylopectin"	Residue
Yield	1.3 gm.	0.1 gm.	0.1 gm.
B.V.	1.01	0.21	-

Reprecipitation of an "amylose" fraction using butanol.

Dry powdered amylose (1 gm.; B.V., 0.99, prepared by Method 3 (p. 54)) was added slowly with stirring to water (100 ml.) at 90° saturated with butanol. Stirring was continued for 15 minutes. The procedure of Method 2 was then followed.

Found	"Amylose"	"Amylopectin"	Residue
Yield	0.8-0.9 gm.	0.1	Very small
B.V.	1.06	0.14	-

Preparation of the "amylose" in quantity by precipitation with pyridine.

Dry starch (40 gm.) was fractionated by Method 3. Only a small quantity of the amylose-pyridine complex was broken down by trituration with alcohol. From this, it was calculated that the complex contained 9.0-9.5 gm. crude amylose (B.V.,0.99). The bulk of the amylose-pyridine complex after being washed with water saturated with butanol was subjected to reprecipitation.

Reprecipitation of the crude amylose using butanol (47).

The amylose-pyridine complex was dissolved in a mixture of butanol (100 ml.) and water (900 ml.) and stirred at 90° for ½ hour. The clear solution was cooled to 70°C and filtered

through a large sintered-glass filter (G3). The temperature of the solution was adjusted to 90°. The solution was then poured into a mixture of butanol (400 ml.) and water (3,100 ml.) at 90°, and the combined liquids stirred for 5 minutes at this temperature. The solution was transferred to several Dewar flasks and allowed to cool slowly (60 hours).

The amylose-butanol precipitate was separated at the centrifuge and was washed with water saturated with butanol. A small portion of the precipitate was withdrawn for analysis. The bulk was then submitted to reprecipitation.

Several precipitations were carried out. The results of these were as follows:-

Number of reprecipitations	Uptake of iodine det. by potentiometric titration	Blue Value
0	15.3%	0.99
1	17.1%	1.09
2	18.0%	1.15
3	19.2%	1.20
4	20.1%	1.25
5	20.8%	1.29
6	20.9%	1.30
7 *	20.9%	1.30

*The seventh reprecipitation was carried out with the amylose concentration in the solution being 0.1% instead of 0.2% as for the others.

After the seventh reprecipitation the amylose-butanol complex was not broken down, but was stored in water saturated with butanol. The solution was kept covered with a layer of butanol.

It was calculated that the complex contained 6 gm. amylose. This represents 80% of the amylose present in the starch.

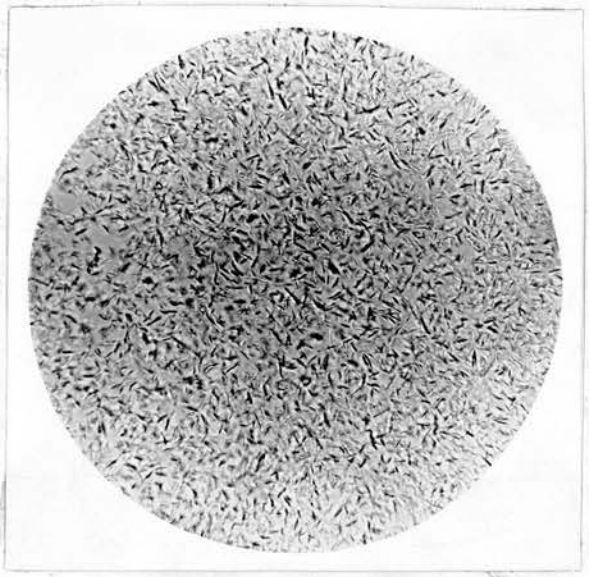
Examination of the amylose-butanol complex.

The amylose-butanol complex was examined under the microscope after each reprecipitation. After the second reprecipitation (iodine uptake, 18.0% - see p. 59), it was observed that the complex appeared similar to the corn amylose-butanol complex (see (27)), taking the form of six-segmented particles. It was observed that if the complex was allowed to stand exposed to the atmosphere for any length of time, the segmented particles became circular (approx. diameter 5-15 μ) and generally lost their finite shape, probably due to the loss of butanol by volatilisation. This was also observed by Schoch (27) for the corn amylose-butanol complex.

After the third reprecipitation, it was found that the shape of the particles had altered. They now appeared in the form of rectangular platelets and showed similarity to the corn amylose-butanol complex found by Kerr and Severson (38).

After the fourth reprecipitation, it was found that the shape of the particles had again altered. The complex now appeared in the form of short thin needles (see Plate 1). This

PLATE 1



(MAGNIFICATION = 400)

appeared similar to the potato and tapioca amylose-butanol complexes found by Kerr (51,73). The complex retained this shape even after four more reprecipitations.

INVESTIGATION OF THE AMYLOSE.

The butanol-amylose complex dissolved easily in warm water (50°) to give a clear solution which was coloured an intense blue on the addition of a drop of iodine solution (0.1N).

After standing for 2-3 days, the clear amylose solutions became cloudy and finally, after 7 days' standing, a precipitate of retrograded amylose could be centrifuged off.

$[\alpha]_D^{18}$ = + 205° (C = 0.5% in water)

$[\alpha]_D^{18}$ = + 149° (C = 0.5% in NaOH (N))

$[\alpha]_D^{18}$ = + 200° (C = 0.5% in perchloric acid (30%)).

Preparation of the amylose for analysis from the complex with butanol.

A quantity of the amylose-butanol complex was dissolved in warm water and made up to a standard volume. An aliquot of this solution was evaporated to dryness under reduced pressure, and the weight of amylose determined directly.

Hydrolysis of the amylose with sulphuric acid.

Amylose (0.0988 gm.) was hydrolysed by boiling with sulphuric acid (2%, 25 ml.) for 7 hours. The resultant glucose was estimated by the method described on p. 49 using alkaline hypoiodite.

Wt. of glucose found = 0.1075 gm.

This corresponds to 97.9% of the theoretical yield of glucose from the amylose.

Methylation of Amylose.

The amylose was methylated under conditions similar to those used by Hirst and Young (19) for rice starch. Amylose solution (50 ml.; containing 4.5 gm. polysaccharide) was mixed with sodium hydroxide (25 ml.; 30%) and then dimethyl sulphate (50 ml.) and sodium hydroxide (100 ml.; 30%) were added gradually at room temperature with vigorous mechanical stirring, in an atmosphere of nitrogen. After 15 hours' stirring, the alkali was partially neutralised with sulphuric acid (30 gm.; 50%). The solution was evaporated on the steam-bath, and the product (without separation of the mineral salts) was remethylated with dimethyl sulphate (35 ml.) and sodium hydroxide (90 ml.;30%). After 15 hours' stirring at room temperature, acetone (125 ml.) was added and the mixture was evaporated on the steam-bath. The insoluble methylated amylose was removed from the surface of the mixture, dissolved in acetone (100 ml.) and water (25 ml.) and remethylated with dimethyl sulphate (35 ml.) and sodium hydroxide (90 ml.; 30%). After this methylation procedure had been repeated a further 11 times, a product was obtained, the methoxyl value of which was not raised on further methylation.

After isolation, the product was purified by washing with boiling water until free from sulphate. It was then dissolved in chloroform and the solution dried with anhydrous sodium sulphate. The methylated amylose was precipitated by pouring the chloroform solution (100 ml.) into light petroleum (1 litre; b.r. 40-60).

Yield, 3.8 gm. (67% of theoretical) OMe, 44.7%.

The methylated amylose was obtained as a white solid which formed rubber-like films which could not be powdered.

Fractionation of methylated amylose.

The methylated amylose (3.8 gm.) was dissolved in chloroform (50 ml.) and light petroleum (b.r. 40°-60°) was slowly added. After the addition of 175 ml. petroleum, precipitation took place, yielding an oil (fraction A) which hardened to yield a white solid after decantation of the supernatant liquid and the addition of fresh petroleum solution.

On the addition of more light petroleum (25 ml.) to the chloroform solution, precipitation again took place (fraction B). A white solid was again obtained.

No further precipitation took place after the addition of more light petroleum (total volume, 600 ml.). A residue (fraction C) was obtained on evaporation of the chloroform-light petroleum mixture to dryness.

Found	Fraction A	2.7 gm.	OMe, 44.8%	$[\alpha]_D^{20}$ + 206°
	Fraction B	0.8 gm.	OMe, 44.9%	$[\alpha]_D^{20}$ + 204°
	Fraction C	0.2 gm.	OMe, 21.7%	(C, 0.5% in chloroform in each case)

(Fraction C was not investigated further).

Viscosity measurements of methylated amylose fractions.

The viscosities of the methylated amylose fractions were determined in m-cresol in an Ostwald viscometer at 20°C. The results were as follows:-

	Fraction A.	Fraction B.
Conc. of amylose in solution	0.4%	0.4%
Average time of flow in secs. Solution (T_1)	1165	973.7
Solvent (T)	416.8	
Specific viscosity, η_{sp} i.e. $\frac{T_1 - T}{T}$	1.80	1.34
η_{sp}/c	0.45	0.335
Molecular Weight (M)	92,000	67,500
Degree of Polymerisation	450	330

The molecular weights were calculated from the equation $\eta_{sp} = K_m M c$ where C is the concentration in gm.-mol. of repeating units per litre. The constant, K_m, was assumed to be that for the methylated cellulose, i.e., 10×10^{-4} (see ref. 90).

Hydrolysis of the methylated amylose on a small scale and examination of the products by paper chromatography (77).

Fractions A and B (50 mgm.) were separately treated with methanolic hydrogen chloride (1 ml.; 4%) in sealed tubes at 100° for 5 hours. After hydrolysis, the tubes were cautiously opened. After removal of the solvent, the residual syrups were hydrolysed for 7 hours with hydrochloric acid (5 ml.; 4%), neutralised with silver carbonate, and filtered. Silver was then removed from the filtrate by hydrogen sulphide and basic or acid ions by treatment with "Amberlite" resins 1R100 and 1R4B. The clear solutions were then concentrated to thin syrups at 40° under reduced pressure. They were then examined by paper chromatography. Development of the chromatogram was carried out with aniline oxalate (107). The results showed that hydrolysed fraction A contained 2:3:6-trimethyl glucose (R_G, 0.81-0.82) in quantity and 2:3-dimethyl glucose (R_G, 0.57-0.58) and another dimethyl sugar (R_G, 0.50-0.53), probably 2:6-or 3:6-dimethyl glucose, in smaller concentration.

Hydrolysed fraction B contained a trace of 2:3:4:6-tetramethyl glucose (R_G, 1.0) and 2:3:6-trimethyl glucose in quantity (R_G, 0.81-0.82).

Hydrolysis of methylated amylose fraction A.

Fraction A (2.65 gm.) was hydrolysed by boiling with methanolic hydrogen chloride (150 ml., 1%) until the rotation was constant (7 hours). The solution was then cooled and the contents cautiously neutralised with silver carbonate. The silver chloride was filtered off and washed well with hot dry methanol. Excess silver was removed by the passage of hydrogen sulphide through the solution, and the insoluble silver sulphide was filtered off. The filtrate was then evaporated to a syrup. Yield, 2.93 gm. (96% of theoretical yield of glycosides).

The syrupy glycosides were hydrolysed by boiling with hydrochloric acid (100 ml., 2%) until the rotation was constant (7 hours). The solution was cooled and the contents neutralised with silver carbonate as described above. On evaporation of the final solution to dryness, a syrup was obtained which partially crystallised on standing. Yield, 2.63 gm. (91% of theoretical yield from the polysaccharide).

Separation of the methylated glucoses on a cellulose column.

A column of powdered cellulose (50 x 3.5 cm.) was prepared, washed and tested as described by Hough, Jones and Wadman (79). The solvent employed for elution was a mixture of purified light petroleum (b.r. 100°-120°) (50%) - n-butanol (50%), saturated with water. The mixture of methylated glucoses obtained above (2.63 gm.) was dissolved in the minimum volume

of the solvent and the solution added dropwise to the centre of the top of the column, each drop being allowed to soak in before the next drop was added. After allowing the column to stand for two hours, the reservoir was filled with solvent and the elution process began. An automatic device changed the receiving tube every six minutes.

The eluate from every tenth tube was concentrated on a watch-glass over a water-bath and examined by paper chromatography in the usual way. The residues on the watch-glasses were dissolved in acetone and transferred quantitatively to the respective tubes.

450 tubes were obtained, each containing 4-5 ml. solvent. Tubes 60-100 were found to contain a sugar corresponding to 2:3:6-trimethyl glucose. The presence of no other sugar was indicated.

Groups of 5 tubes were then combined, concentrated, and examined by paper chromatography. Tubes 31-50 were found to contain 2:3:4:6-tetramethyl glucose, and tubes 56-60 and 101-110 were found to contain 2:3:6-trimethyl glucose.

The tubes were then suitably combined, the solvent was removed at 40°/20 mm., and the residue dissolved in water and filtered through charcoal to remove waxy impurities. After further concentration, the residue was dissolved in acetone and the last traces of waxy impurities were removed by filtration.

The column was then washed with water (1 litre). The aqueous extract was evaporated to dryness under reduced pressure and waxy materials were removed from the residue as above.

Examination by paper chromatography showed that dimethyl glucoses (see later) were principally present.

Three fractions were therefore obtained -

1)	Tetramethyl glucose,	0.022 gm.
2)	Trimethyl glucose,	2.217 gm.
3)	Dimethyl glucose,	0.039 gm.
	Total	2.278 gm. (Recovery, 86%)

EXAMINATION OF THE FRACTIONS.

1) Tetramethyl glucose fraction.

Chromatographic examination indicated the presence of a single substance (R_G, 1.0) which corresponded to 2:3:4:6-tetramethyl glucose.

The syrup (0.022 gm.) was dissolved in water (12.5 ml.) A portion of this solution (2.5 ml.) was hydrolysed by boiling with sulphuric acid (1 ml.; 2%) for 6 hours. The acid was neutralised with barium carbonate and the barium sulphate was filtered off and washed. The filtrate was concentrated and examined by paper chromatography. The presence of 2 substances was indicated. One (R_G, 1.0) corresponded to 2:3:4:6-tetramethyl glucose, whilst the other (R_G, 0.82) corresponded to 2:3:6-trimethyl glucose.

Quantitative estimation of the mixture by the method of Hirst, Hough and Jones (77, see below) revealed the presence of 33.7% of tetramethyl glucose.

The method of Hirst, Hough and Jones for the estimation of methylated sugars by alkaline hypoiodite after separation on the paper chromatogram was modified by the use of a sodium hydroxide-phosphate buffer (pH, 11.4) (108) in place of the

carbonate-bicarbonate buffer (pH, 10,6).

Quantitative estimation of the tetramethyl glucose by alkaline hypoiodite oxidation.

Two portions (5 ml. each) of the dissolved fraction (see above) were treated with iodine (1 ml. 0.1N) measured from an "Agla" micrometer syringe (in two batches) and buffer solution (2 ml.; pH, 11.4, see above) in "Quickfit" boiling tubes which were sealed with stoppers moistened with potassium iodide solution (10%). The tubes were kept in a cool, dark place for 4 hours, the stoppers washed with water, and the solutions acidified with sulphuric acid (2 ml.; 2N) and titrated with sodium thiosulphate (0.01N). A blank was run concurrently. Results were as follows:-

Solution	Vol. of thiosulphate used to titrate solution (ml.)	Vol. of iodine required to oxidise sugar (ml.)	Weight of sugar oxidised (mgm.)
Blank	9.320	-	-
1	7.258	2.062	2.56
2	7.161	2.159	2.68

Normality of thiosulphate, 0.0105.

These results correspond to the presence of one non-reducing terminal group per 400±40 glucose residues.

Quantitative estimation of trimethyl glucose in the tetramethyl glucose fraction.

The solutions obtained after estimation of the tetramethyl glucose (see above) were combined and neutralised with barium carbonate. After filtration, the solution was evaporated to dryness and the residue was extracted several times with chloroform. The chloroform extracts were evaporated to dryness,

and the residue was then hydrolysed with sulphuric acid (2 ml.; 2%) for 6 hours. After neutralisation, the solution was examined by paper chromatography. The presence of a single substance (R_G, 0.81) corresponding to 2:3:6-trimethyl glucose, was indicated. On testing with silver nitrate, the solution was found to be free from iodine. Portions (5 ml.) were oxidised with alkaline hypoiodite as described above. 12.95 mgm. trimethyl glucose was found to be present in the fraction.

2) Trimethyl glucose fraction (2.217 gm.)

Examination of this fraction by paper chromatography indicated the presence of a single substance corresponding to 2:3:6-trimethyl glucose. Hydrolysis of a portion of this fraction with sulphuric acid (2%) and re-examination by paper chromatography did not reveal the presence of any other sugar. Hypoiodite oxidation by the method described before, indicated that the sugar was 98% pure.

The fraction crystallised almost completely on standing. The material (2.15 gm.) was recrystallised twice from dry ether.

Yield, 1.51 gm. M.P., 115-117° alone or when mixed with an authentic specimen of 2:3:6-trimethyl D-gluco-pyranose.
OMe, 41.4% (theoretical 41.9%).

$[\alpha]_D^{18}$, + 94.3° → + 71.6° (C = 1% in water).

Rotation of 2:3:6-trimethyl glucose in cold methanolic hydrogen chloride solution

2:3:6-trimethyl glucose (0.101 gm.) was dissolved in methanolic hydrogen chloride (10 ml.; 2%) at room temperature, and the rotation of the solution was observed at intervals using

a 2 dm. tube. Results were as follows:-

Time (hrs.)	Observed Rotation (α) ^{o}	$[\alpha]_{D}^{18}$
0	+ 1.40	+ 69.3°
1	+ 0.65	+ 32.2°
2	+ 0.08	+ 4.0°
3	- 0.29	- 14.4°
5	- 0.57	- 28.2°
7	- 0.69	- 34.2°
9	- 0.69	- 34.2°
24	- 0.69	- 34.2°

3) Dimethyl glucose fraction (0.039 gm.)

Chromatographic examination revealed the presence of the following sugars:- 2:3-dimethyl glucose (R_G, 0.57) - in small concentration. 2:6- or 3:6-dimethyl glucose (R_G. 0.51) or a mixture of both (they are not separated by paper chromatography) - in quantity. A monomethyl glucose (R_G, 0.26) - trace. Glucose (R_G, 0.10) - trace.

Quantitative examination of the mixture of dimethyl sugars revealed the presence of 2.6% of the 2:3 isomer. This corresponds to 1.02 mgm. of 2:3-dimethyl glucose in the fraction.

OMe, 28.9% (calculated for dimethyl hexose 29.7%).

$[\alpha]_{D}^{18}$, + 62.3° at equilibrium (C = 0.5% in water).

Rotation of the dimethyl glucose fraction in cold methanolic hydrogen chloride solution.

The dimethyl glucose fraction (0.035 gm.) was dissolved in methanolic hydrogen chloride solution (10 ml., 2%) at room temperature, and the rotation was observed at intervals as above. In 6 hours it was found that the rotation underwent the following change:-

$[\alpha]_{D}^{18}$, + 60.3° (initial) $\longrightarrow$ - 10.0° (final)

Estimation of 2:6-dimethyl glucose present in the dimethyl fraction by the method of Bell (80) using sodium metaperiodate.

The solution of the dimethyl fraction (0.035 gm.) in methanolic hydrogen chloride (see p. 70) was heated at 100° for 3 hours. A slight excess of saturated sodium bicarbonate was added and the alcohol was evaporated on the steam-bath. Water (2.1 ml.) was then added to the residue. The solution (0.1 ml.) was found to be non-reducing on testing with Fehling's solution. The pH was adjusted to 7 (bromothymol blue) by dilute acetic acid and sodium metaperiodate (2 ml.; 0.3M) was added. After 5 hours at room temperature, the amount of periodate consumed was determined by titration with sodium arsenite (0.05N) after the addition of phosphate buffer (2 ml.; 0.5M, pH 7.5) and excess potassium iodide.

It was found that the uptake of sodium metaperiodate was 0.84 moles per dimethyl methyl-glucoside unit. This indicated the presence of 84% of 2:6-dimethyl glucose in the dimethyl fraction.

Confirmation of the presence of 3:6-dimethyl glucose in the dimethyl fraction.

After estimation of the periodate uptake (see above), ethylene glycol (1 ml.) was added to the solution to destroy excess periodate. The solution was then evaporated to dryness and the residue extracted several times with chloroform. The chloroform extracts were evaporated to dryness and the residue was hydrolysed with sulphuric acid (2 ml.; 2%) for 6 hours. After neutralisation, the solution was examined by paper

chromatography. Two substances were found to be present. One (R_G, 0.57) corresponded to 2:3-dimethyl glucose, whilst the other (R_G, 0.51) corresponded to 3:6-dimethyl glucose.

Hydrolysis of methylated amylose fraction B.

Fraction B (0.726 gm.) was hydrolised to glycosides and then to reducing sugars, as described on p. 65.

Yield of glycosides, 0.812 gm. (97% of theoretical).
Yield of reducing sugars, 0.737 gm. (93% of theoretical).

Separation of the methylated sugars on a cellulose column.

The method and the solvents were the same as described before (p. 65) for fraction A.

The syrup (0.737 gm.) was dissolved in the minimum volume of light-petroleum (b.r. 100-120°) - n-butanol (5:5), saturated with water, and the solution was gradually added to the well-washed cellulose column (50 x 3.5 cm.). The column was developed with the light petroleum-butanol mixture and the eluate was collected in a series of tubes, each containing 4-5 ml. solvent. A total of 270 tubes were obtained. The contents of every tenth tube were concentrated and examined by paper chromatography. Tubes 191-220 were found to contain 2:3:6-trimethyl glucose. The presence of no other sugar was indicated. Groups of 5 tubes were then combined, concentrated and examined by paper chromatography. Tubes 91-100 were found to contain 2:3:4:6- tetramethyl glucose.

The tubes were than suitably grouped, the solvent was removed under reduced pressure, and waxy impurities were removed.

The column was then washed with water (1 litre) and the

aqueous extract was evaporated and purified. Chromatographic examination showed that it principally contained dimethyl glucoses. Three fractions were therefore obtained.

1) Tetramethyl glucose	0.010 gm.	
2) Trimethyl glucose	0.616 gm.	
3) Dimethyl glucose	0.007 gm.	
Total	0.633 gm.	(Recovery, 86%).

EXAMINATION OF THE FRACTIONS.

1) Tetramethyl glucose fraction.

Chromatographic examination indicated the presence of a single substance (R_G, 1.0) which corresponded to 2:3:4:6-tetramethyl glucose.

The syrup (0.010 gm.) was dissolved in water (12.5 ml.). A portion of this solution (2.5 ml.) was hydrolysed by boiling with sulphuric acid (1 ml.; 2%) for 6 hours. The acid was neutralised with barium carbonate and the barium sulphate was filtered off and washed. The filtrate was concentrated and examined by paper chromatography. The presence of 2 substances was indicated. One (R_G, 1.0) corresponded to 2:3:4:6-tetramethyl glucose, whilst the other (R_G, 0.82) corresponded to 2:3:6-trimethyl glucose.

Quantitative estimation of the mixture as before (p. 67) revealed the presence of 39.6% of tetramethyl glucose.

Quantitative estimation of the tetramethyl glucose by alkaline hypoiodite oxidation.

Two portions (5 ml. each) of the dissolved fraction (see above) were oxidised by treatment with alkaline hypoiodite, as described on p. 68. The following results were obtained:-

Solution	Vol. of thiosulphate used to titrate solution (ml.)	Vol. of iodine required to oxidise sugar (ml.)	Weight of sugar oxidised (mgm.)
Blank	9.320	-	-
1	8.254	1.066	1.32
2	8.200	1.120	1.39

Normality of thiosulphate, 0.0105.

These results correspond to the presence of one non-reducing terminal group per 215± 25 glucose residues.

Quantitative estimation of trimethyl glucose in the tetramethyl glucose fraction.

The solutions obtained after estimation of the tetramethyl glucose were combined and neutralised, as described for fraction A (see p. 68). After hydrolysis, the solution was examined by paper chromatography. The presence of a single substance (R_G, 0.82) corresponding to 2:3:6-trimethyl glucose was indicated. Portions (5 ml.) of the solution were oxidised with alkaline hypoiodite, as described before. 5.42 mgm. trimethyl glucose was found to be present in the fraction.

2) Trimethyl glucose fraction (0.616 gm.).

Examination of this fraction by paper chromatography indicated the presence of a single substance corresponding to 2:3:6-trimethyl glucose. Hydrolysis of a portion of this fraction with sulphuric acid (2%), and re-examination by paper chromatography, did not reveal the presence of any other sugar. Hypoiodite oxidation by the method described before indicated that the sugar was 98% pure.

The fraction partially crystallised on standing. The material (0.52 gm.) was recrystallised twice from dry ether.

Yield, 0.24 gm. M.P., 114-116° alone or when mixed with an authentic sample of 2:3:6-trimethyl glucopyranose or the trimethyl glucose sample described on p. 69.
OMe, 41.4% (calculated 41.9%).

$[\alpha]_D^{20}$, + 91.5° → +69.9° (C = 1% in water).

Rotation of 2:3:6-trimethyl glucose in cold methanolic hydrogen chloride solution.

2:3:6-trimethyl glucose (0.100 gm.) was dissolved in methanolic hydrogen chloride solution (10 ml.; 2%) at room temperature, and the rotation was observed at intervals as before (p. 70). In 7 hours it was found that the rotation underwent the following change:-

$[\alpha]_D^{20}$, + 68.7° (initial) ⟶ - 35.1° (final)

3) Dimethyl fraction (o.007 gm.).

Chromatographic examination revealed the presence of the following sugars:-

2:3-dimethyl glucose (R_G, 0.57)
2:6- or 3:6- dimethyl glucose (R_G, 0.51) (see p. 70).

Traces of a monomethyl glucose (R_G, 0.26) and glucose (R_G, 0.09) were also observed.

Estimation of the dimethyl glucoses.

Chromatographically pure 2:3:6-trimethyl glucose (15.09 mgm.) was added to the dimethyl fraction as a reference sugar, and the components were estimated by the method of Hirst, Hough and Jones (see p. 67).

0.70 mgm. 2:3-dimethyl glucose was found to be present.
1.31 mgm. 2:6- or 3:6-dimethyl glucose was found to be present.

Demethylation of 2:3:4:6- tetramethyl and 2:3:6- trimethyl glucose.

Chromatographically pure 2:3:4:6- tetramethyl glucose

(50 mgm.; M.P. 85-86°) and 2:3:6- trimethyl glucose (50 mgm.; M.P. 115-117°) were separately heated in sealed tubes with methanolic hydrogen chloride (1 ml.; 1%) for 7 hours at 100°. On cooling, the tubes were opened and the acid was neutralised with silver carbonate. After filtration, the solutions were evaporated to dryness. The residues were then rehydrolysed with hydrochloric acid (2.5 ml.; 1%) by heating at 100° for 7 hours. The acid was neutralised with silver carbonate and the sugars were regenerated as described before (p. 67), finally being obtained as syrups. Chromatographic examination indicated the presence of a very small proportion of trimethyl glucose (R_G, 0.82) and faint traces of dimethyl and monomethyl glucoses along with the tetramethyl glucose.

Quantitative estimation of the mixture (see p. 64) revealed the presence of 1% of trimethyl glucose.

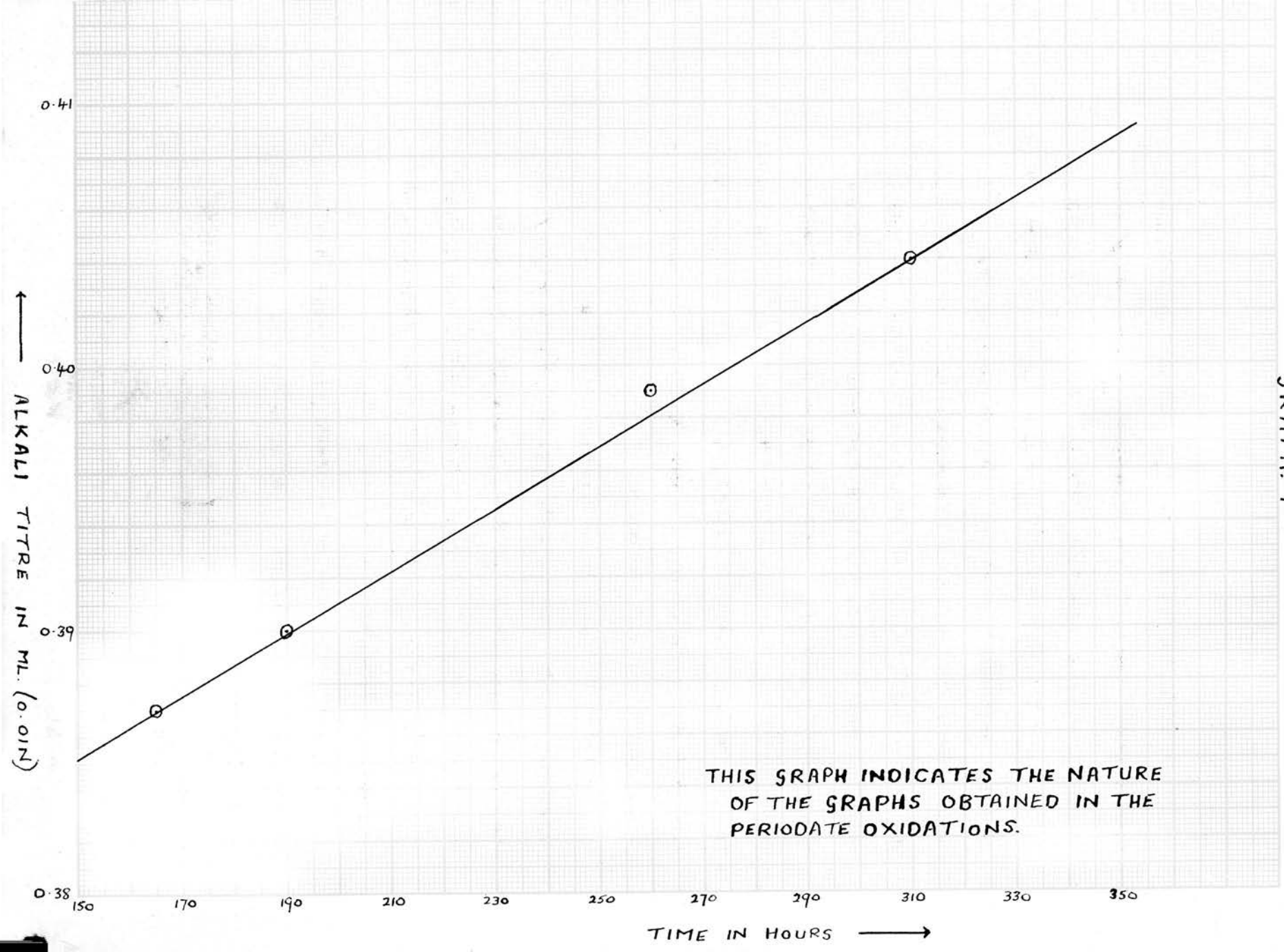
With the trimethyl glucose, chromatographic examination indicated the presence of very small proportions of 2 dimethyl glucoses corresponding to 2:3-dimethyl glucose (R_G, 0.58) and 2:6- or 3:6-dimethyl glucose (R_G, 0.52) and a trace of monomethyl glucose.

Quantitative estimation of the mixture (see p. 67) revealed the presence of 0.7% of dimethyl glucoses. No attempt was made to estimate the dimethyl components separately as the figure was so low.

Periodate Oxidation of Amylose.

Estimation of formic acid liberated from the end-groups .

The method of Brown, Halsall, Hirst and Jones (41) was used



GRAPH. 1

THIS GRAPH INDICATES THE NATURE OF THE GRAPHS OBTAINED IN THE PERIODATE OXIDATIONS.

as follows:-

Amylose (0.2441 gm.; freshly prepared from the complex with butanol - see p. 61) was dissolved in water (52.5 ml.) containing potassium chloride (2.5 gm.) and sodium metaperiodate (7.5 ml.; 0.3M) was added. The mixture was shaken continuously in a stoppered bottle completely surrounded with black cloth. At intervals the shaking was stopped and the solid matter was allowed to settle out. Samples (10 ml.) of the clear supernatant liquid were then withdrawn and ethylene glycol (3 drops) was added with shaking. After standing for 1 hour, the solutions were titrated with sodium hydroxide (0.01N) from a microburette, using methyl red as indicator. After the samples had been withdrawn, the shaking was again started. A blank was run concurrently with the experiment.

The experimental titres, apart from the first, were corrected to allow for the fact that the oxidised amylose was not removed from the bottle when a sample of liquid was withdrawn for titration. This was confirmed by combining two of the samples used for titration, dialysing and evaporating the dialysate to dryness. No residue was found.

The corrected titres were plotted against time (see Graph 1) and the acid yield after 150 hours oxidation was read. Results were as follows:-

Time of shaking (hours)	Volume of sodium hydroxide (0.01N) (corrected) used per 10 ml. sample withdrawn. (ml.)
165	0.387
190	0.390
260	0.399
310	0.404

From Graph 1 it was found that the titre after 150 hours would be 0.385 ml. sodium hydroxide. Therefore, 162 gm. amylose would yield 0.01535 moles formic acid. This corresponds to the yield of one mole formic acid per 65 glucose units or to the presence of one non-reducing terminal group per 195 glucose residues.

The experiment was repeated. Results were as follows:-

Wt. of amylose, 0.2337 gm.

Total volume of solution, 60 ml.

10 ml. samples withdrawn.

Time of shaking (hours)	Corrected volumes of sodium hydroxide (0.01N) used per 10 ml. sample. (ml.)
165	0.308
190	0.309
260	0.319
310	0.323
Estimated titre after 150 hours' shaking	0.306 ml.
Yield of formic acid (moles) from 162 gm. amylose	0.01273
No. of glucose residues per non-reducing terminal group	234

The average of the two results indicates the presence of one non-reducing terminal group per 215 ± 20 glucose units.

Hydrolysis of the amylose after oxidation with periodate.

After the last sample of liquid had been withdrawn from sample 1 (see above), ethylene glycol (1.5 ml.) was added to destroy excess periodate, by shaking overnight. The oxidised

amylose was then dialysed until free from oxidant (test with potassium iodide and sulphuric acid and diphenylamine and sulphuric acid). The solution was then reduced in volume (to 100 ml.).

Sodium metaperiodate (15 ml.; 0.3M) was then added to the solution, and the periodate uptake was determined at intervals in the following way:-

Portions (1 ml.) were withdrawn, and to each of these sodium arsenite (5 ml.; 0.1N) was added. The reagents were mixed and allowed to stand for 15 minutes. The excess arsenite was titrated against iodine (0.1N) using starch as indicator. The oxidation was carried out at room temperature.

Results showed that the periodate uptake was negligible, and that the amylose appeared to be completely oxidised.

The excess periodate was decomposed by shaking with ethylene glycol as before, and the oxidised amylose dialysed until free from oxidant (see above). The solution was then evaporated to dryness and the residue was hydrolysed with sulphuric acid (50 ml.; 0.5N) by heating at 95° for 10 hours. The acid was neutralised with barium carbonate, and the barium sulphate was filtered off and washed. The filtrate was evaporated to dryness. Only a very small white residue remained. This was dissolved in water (0.1 ml.) and the solution was examined by paper chromatography. No sugar was found to be present.

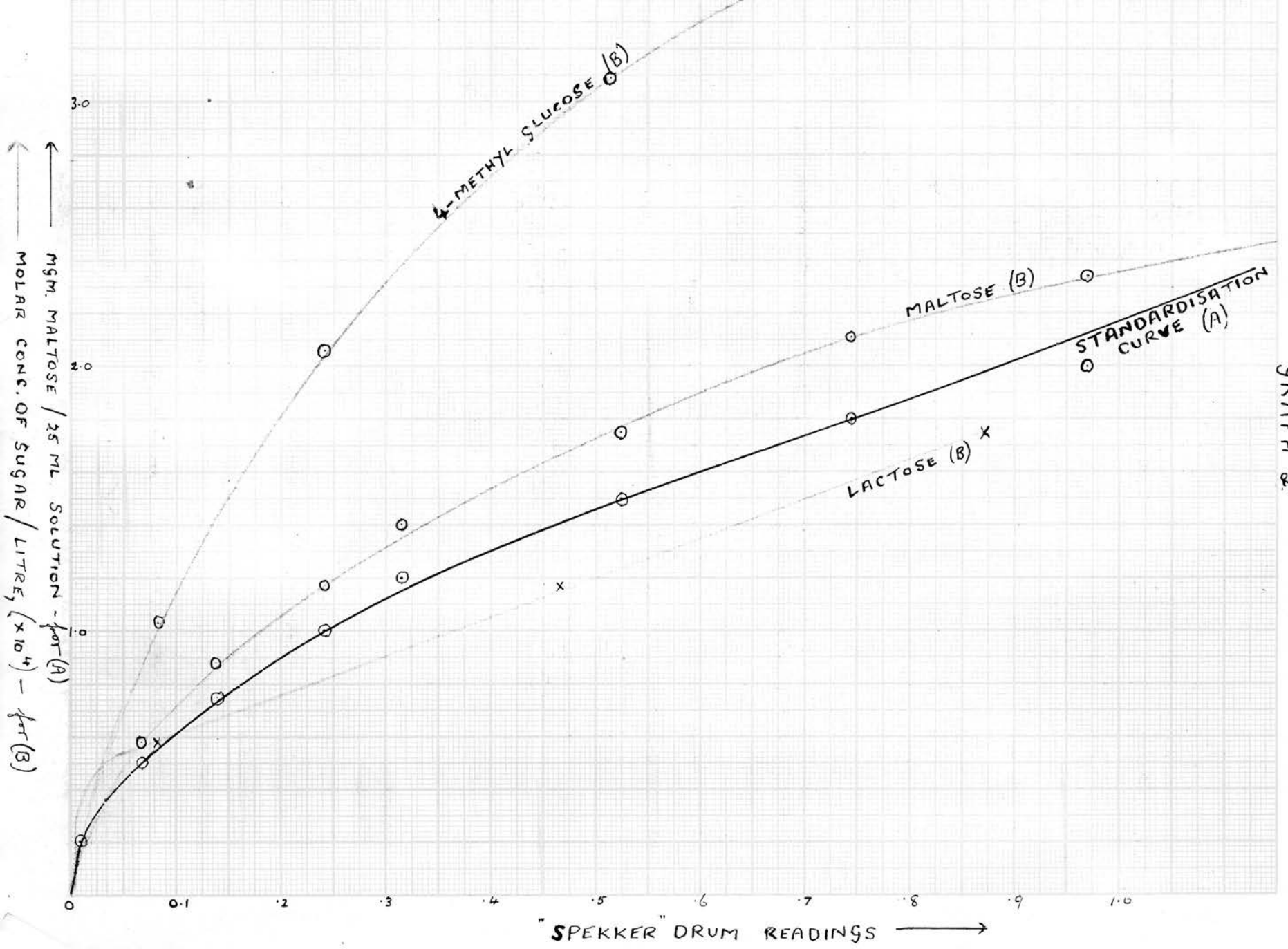
Determination of the degree of polymerisation of amylose by the method of Meyer, Noelting and Bernfeld (66) using 3:5-dinitrosalicylic acid.

Reagents - solution A: 3:5-dinitrosalicylic acid (1.5%)
solution B: sodium hydroxide (6N).

Preparation of standardisation curve using maltose. Maltose (2 ml., containing 0.1-2.0 mgm.) was diluted with water (1 ml.) and added to solution A (1 ml.) and solution B (1 ml.). A reagent blank was also prepared. The combined solutions were heated for 30 minutes at 65°, cooled and diluted with water to a standard volume (25 ml.). The reduced solution was compared against the blank in a "Spekker" photoelectric absorptiometer, using 4 cm. cells and green filters (No. 604). The results for the standardisation curve were as follows:-

Mgm. Maltose in 25 ml. solution	Molar concentration per litre ($\times 10^{-4}$)	"Spekker" Drum reading using 4 cm. cell
0.2	0.23	0.009
0.5	0.58	0.067
0.75	0.88	0.138
1.0	1.17	0.241
1.2	1.40	0.315
1.5	1.75	0.524
1.8	2.11	0.745
2.0	2.34	0.970

Estimation of Degree of Polymerisation. Amylose solution (3 ml. containing about 60 mgm. polysaccharide was added to solution A (1 ml.) and solution B (1 ml.). The mixture was heated at 65° for 30 minutes, cooled and diluted with water to standard volume (25 ml.). The blank solution consisted of amylose solution (3 ml. as above), water (1 ml.) and solution B (1 ml.). This was heated at 65° for 30 mimutes, cooled, solution A (1 ml.) added, and then diluted to standard volume (as above). The two solutions were then compared in the "Spekker" absorptiometer as before. Results were as follows:-


4-METHYL GLUCOSE (B)
MALTOSE (B)
STANDARDISATION CURVE (A)
LACTOSE (B)
3·0
2·0
1·0
0
0·1
·2
·3
·4
·5
·6
·7
·8
·9
1·0
MGM. MALTOSE / 25 ML. SOLUTION - for (A)
MOLAR CONC. OF SUGAR / LITRE, (×10⁴) - for (B)
"SPEKKER" DRUM READINGS

GRAPH 2.

	WT. of AMYLOSE TAKEN (MGM.)	"SPEKKER" DRUM READING, USING 4 CM. CELL	EQUIV. WT. of MALTOSE from GRAPH 2.	DEGREE of POLYMERI-SATION	CALCULATED MOLECULAR WEIGHT
1	50.14	0.144	0.76	132	21,500
2	50.77	0.112	0.67	152	24,500
3	53.83	0.198	0.90	120	19,500
4	55.29	0.141	0.75	147	24,000
5	59.28	0.223	0.96	123	20,000

These results show that amylose has an apparent degree of polymerisation of 135±20 glucose units corresponding to a molecular weight of 22,000±3,000.

Comparison of reaction of other 4-substituted glucose derivatives with 3:5-dinitrosalicylic acid.

Lactose and 4-methyl glucose (0.5-1.5 mgm.) were treated with 3:5-dinitrosalicylic acid as described for maltose (p. 80), and the solutions were compared in the "Spekker" in the usual way. Results were as follows:-

Sugar	Mgm. present in 25 ml. solution	Molar concentration per litre ($\times 10^{-4}$)	"Spekker" drum reading using 4 cm. cells
Lactose	0.5	0.58	0.082
	1.0	1.17	0.465
	1.5	1.75	0.873
4-methyl glucose	0.5	1.03	0.061
	1.0	2.06	0.241
	1.5	3.09	0.514

The results were compared with those of maltose (see Graph 2).

AMYLOPECTIN FRACTION.

Attempted removal of amylose from the "amylopectin" fraction.

The amylopectin fractions obtained from the various precipitations (see p. 51-56) were dispersed as before and the solutions were again treated with the respective precipitants.

In no case was there any significant precipitation.

Preparation of the amylopectin in quantity.

This was carried out by Method 4B (p. 56) using pyridine and butanol. 20 gm. starch was dispersed.

Yield of amylopectin, 9.0 gm. B.V., 0.067.
Uptake of iodine by potentiometric titration, 0.43%.
Amylose = 2%.

A total of 11.1 gm. amylopectin was therefore available for investigation.

INVESTIGATION OF THE AMYLOPECTIN.

The white solid dispersed easily in hot water on stirring. The solution gave a blue coloration with one drop of iodine (0.1N). The addition of further iodine yielded a reddish-purple coloration.

$[\alpha]_D^{15}$ = +168° (C = 0.76% in perchloric acid (30%)).
$[\alpha]_D^{15}$ = +149° (C = 0.75% in NaOH (N)).

Methylation of Amylopectin.

This was carried out by the method used for amylose (see p. 62). The amylopectin (9.1 gm.) was dispersed in cold water (75 ml.) and sodium hydroxide (50 ml.; 30%) was added. The thick gel was partially dispersed by the addition of more water (25 ml.). Sodium hydroxide (200 ml.; 30%) and dimethyl sulphate (100 ml.) were then added gradually at room temperature with vigorous mechanical stirring. A brisk reaction took place, and the containing vessel was cooled to prevent a sudden rise in temperature. After 15 hours' stirring, the alkali was partially neutralised with sulphuric acid (60 gm.; 50%) and the mixture was methylated again in aqueous solution and then 4 times in an

aqueous acetone solution as described on p. 62.

The product was isolated in the usual way and was purified by washing with boiling water until free from sulphate. It was then dissolved in chloroform and the solution dried with anhydrous sodium sulphate. The methylated amylopectin was purified by pouring the chloroform solution (200 ml.) into light petroleum (2 litres; b.r. 40-60°). Yield, 7.3 gm. (64% of theoretical). OMe, 44.0%.

The methylated amylopectin was obtained as a white solid which was easily powdered.

Fractionation of methylated amylopectin.

The methylated amylopectin (7.3 gm.) was dissolved in chloroform (100 ml.) and light petroleum (b.r. 40-60°) was slowly added with stirring. After the addition of 350 ml. light petroleum, precipitation took place, yielding an oil (fraction A) which hardened to yield a white solid after decantation of the supernatant liquid and the addition of fresh petroleum solution. On the addition of more petroleum (100 ml.) to the chloroform solution, precipitation again took place (fraction B). A white solid was again obtained.

No further precipitation took place after the addition of more petroleum (total volume, 1 litre). A residue was obtained on evaporation of the chloroform-light petroleum mixture to dryness.

Found	fraction A,	5.6 gm.	OMe, 44.0%.	$[\alpha]_D^{17}$, + 204°
	fraction B,	1.5 gm.	OMe, 43.9%.	$[\alpha]_D^{17}$, + 204° (C =
	residue,	0.2 gm.	OMe, nil.	0.5% in chloroform in each case).

Viscosity measurements of methylated amylopectin fractions.

The viscosities of the methylated amylopectin fractions were determined in m-cresol in an Ostwald viscometer at 20°C. Results were as follows:-

		Fraction A.	Fraction B.
Conc. of amylopectin in solution (%)		0.4	0.4
Average time of flow in secs.	Solution (T_1)	957.6	677.7
	Solvent (T)	420.6	
Specific viscosity, η_{sp} i.e. $\frac{T_1 - T}{T}$		1.28	0.61
$\frac{\eta_{sp}}{C}$ (C = conc. in gm. /100 ml.)		3.2	1.5

When these figures were referred to the graph obtained by Hirst and Young (19), showing the relationship between η_{sp}/C and the molecular weight for starches, it was found that the apparent molecular weights of the fractions were -

fraction A, 485,000
fraction B, 215,000.

Hydrolysis of methylated fraction A on a small scale and examination of the products by paper chromatography.

Fraction A (50 mgm.) was hydrolysed as described on p. 64. A syrup was finally obtained which was examined by paper chromatography. The components of the mixture were quantitatively estimated by the method of Hirst, Hough and Jones (see p. 67). Results were as follows:-

Sugar indicated	R_G found		Sugar present in mixture %
2:3:4:6-tetramethyl glucose	1.0		4.1
2:3:6-trimethyl glucose	0.81		81.8
2;3-dimethyl glucose	0.56	estimated	12.8
2:6 - dimethyl glucoses	0.51	together	
3:6 - dimethyl glucoses	0.51	together	
Monomethyl glucose	0.25		1.2
Glucose	0.11		trace.

∞

The 2:3-dimethyl glucose appeared to represent about $\frac{1}{3}$ of the dimethyl fraction.

Further methylation of fraction A by the method of Purdie.

Methylated fraction A (4.3 gm.) was dissolved in neutral methyl iodide (100 gm.). The solution was heated to 43° on a water-bath and the temperature was maintained at this level for 24 hours. Dry silver oxide (25 gm.) was added in portions (2 gm.) every half-hour. On cooling, the mixture was diluted (to 200 ml.) with chloroform and filtered. The silver oxide was extracted four times with hot chloroform (150 ml.). The solutions were combined and evaporated to dryness.

After two further treatments as above, the methylated fraction A was found to have a methoxyl content of 45.0% (calculated 45.6%). A further treatment with the reagents did not raise this methoxyl value. Yield, 3.6 gm. (84%).

Attempted fractionation of fully methylated fraction A.

Methylated fraction A (3.6 gm.) was dissolved in chloroform (75 ml.) and light petroleum was added as described before (p. 83). It was found that the fraction was essentially homogeneous.

Viscosity measurements of fully methylated fraction A.

This was carried out as described on p. 84. It was found that the apparent molecular weight of the fully methylated fraction was 520,000.

Hydrolysis of fully methylated fraction A on a small scale and examination of the products by paper chromatography.

Fraction A (50 mgm.) was hydrolysed as described on p. 64. A syrup was obtained which was examined by paper chromatography.

The components of the mixture were quantitatively estimated by the method of Hirst, Hough and Jones (see p. 67). Results were as follows:-

Sugar indicated	R_G found	Sugar present in mixture (%) (average)
2:3:4:6-tetramethyl glucose	1.0	4.2
2:3:6-trimethyl glucose	0.80	90.5
2:3-dimethyl glucose 2:6-dimethyl glucose 3:6-dimethyl glucose	0.55	5.3
Monomethyl glucose	0.26	faint trace
Glucose	0.10	faint trace

The dimethyl sugars were not separated in this case and were estimated together.

Hydrolysis of methylated amylopectin fraction A.

Methylated amylopectin fraction A (3.22 gm.) was hydrolysed to glycosides and then to reducing sugars by the method described (on p. 65) for methylated amylose.

Yield of glycosides, 3.62 gm. (97% of theoretical)
Yield of reducing sugars, 3.25 gm. (92% of theoretical)

The latter was obtained as a pale yellow-coloured syrup.

Separation of methylated sugars on a cellulose column.

The method and the solvents were the same as described (p. 65) for the separation of the sugars from methylated amylose fraction A.

The syrup (3.25 gm.) was dissolved in the minimum volume of light petroleum (b.r. 100-120°)-n-butanol (5:5) saturated with water, and the solution was gradually added to the well-washed cellulose column (80 x 3.3 cm.). The column was developed with the light petroleum-butanol mixture and the eluate was collected in a series of tubes, each containing 4-5 ml. solvent,

after the first 100 ml. had been collected in a flask. A total of 605 tubes was obtained. The contents of every tenth tube were concentrated and examined by paper chromatography. The tubes were then grouped so as to contain only one sugar, and the contents were evaporated and purified as before. The results were as follows:-

Fraction	Tube No.	R_G found	Sugar suspected	Weight (gm.)
	First 100 ml.	-	-	-
	1- 40	-	-	-
1	41- 80	1.0	2:3:4:6-tetramethyl glucose	0.152
	81-160	-	-	-
2	161-210	0.81	2:3:6-trimethyl glucose	2.679
	211-420	-	-	-
3	421-470	0.57	2:3-dimethyl glucose	0.140
	471-490	-	-	-
4	491-540	0.50	2:6 / 3:6 - dimethyl glucose	0.032
	541-605	-	-	-
5	Aqueous extract	0.26 0.09	monomethyl glucose glucose	trace trace
			Wt. of sugar recovered	3.003 gm. (93%)

The column was washed with water (1.5 litres) and the aqueous extract was evaporated and purified. Five fractions were thus obtained.

EXAMINATION OF THE FRACTIONS.

1) Tetramethyl glucose fraction (0.152 gm.)

Chromatographic examination indicated the presence of a single substance (R_G,1.0) which corresponded to 2:3:4:6-tetramethyl glucose. Hypoiodite oxidations on portions of the syrup (2 mgm.) by the method described on p. 68 indicated that

the fraction was 84.9% pure.

The syrup (10 mgm.) was hydrolysed by boiling with sulphuric acid (1 ml.; 2%) for 6 hours. After neutralisation and filtration, the solution was examined by paper chromatography. The presence of 2 substances was indicated. One (R_G, 1.0) corresponded to 2:3:4:6-tetramethyl glucose, whilst the other (R_G, 0.81) corresponded to 2:3:6-trimethyl glucose.

Hydrolysis of fraction 1.

Fraction 1 (0.135 gm.) was hydrolysed by boiling with hydrochloric acid (10 ml.; 1%) for 7 hours. The acid was neutralised with silver carbonate and the silver chloride was filtered off and washed. Excess silver was removed from the solution by the passage of hydrogen sulphide. The silver sulphide was filtered off and washed. The filtrate was evaporated to dryness under reduced pressure. Yield, 0.134 gm.

Separation of the components of hydrolysed fraction 1 on a cellulose column.

The method and solvents were the same as described (p. 65) for methylated amylose fraction A. Hydrolysed fraction 1 (0.134 gm.; see above) was dissolved in the minimum volume of light petroleum-butanol mixture, and the solution was gradually added to the well-washed cellulose column (55 x 1.3 cm.). The column was developed with the mixture and the eluate was collected in a series of tubes, each containing 4-5 ml. solvent, after the first 50 ml. had been collected in a flask. A total of 250 tubes was used. The contents of every tenth tube were concentrated and examined by paper chromatography. The column was washed with water (500 ml.) and the extract concentrated as

before. Three fractions were obtained as indicated below.

Fraction	Tube No.	R_G found	Sugar indicated	Weight (gm.)
	First 50 ml.	-	-	-
	1- 60	-	-	-
1a	61- 90	1.0	2:3:4:6-tetramethyl glucose	0.106
	91-150	-	-	-
1b	151-170	0.82	2:3:6-trimethyl glucose	0.021
	171-250	-	-	-
Aqueous extract	-	0.53	Dimethyl glucose	trace
			Wt. of sugar recovered =	0.127 gm. (95%)

Investigation of fractions 1a and 1b.

Fraction 1a - tetramethyl glucose.

Chromatographic examination indicated the presence of a single substance (R_G, 1.0) which corresponded to 2:3:4:6-tetramethyl glucose. Hydrolysis of this substance with sulphuric acid (2%) and re-examination by paper chromatography did not reveal the presence of any other sugar. Hypoiodite oxidation by the method described on p. 68 indicated that the sugar was 99% pure.

The fraction partially crystallised on standing. The material (95 mgm) was recrystallised twice from purified light petroleum (b.r. 40-60°) and was obtained in the form of long needles which were tiled and washed with petroleum. (All filtrates and washings were retained and evaporated).

Yield of crystalline material, 55 mgm.
M.P., 85-87° alone or admixed with an authentic sample of tetramethyl D-glucopyranose.
OMe, 52.1% (theoretical 52.5%).
$[\alpha]_D^{18}$, + 98.3° ⟶ 83.9° (C = 0.45% in water).

From the weight of "end-group", it was found that the amylopectin possessed 1 non-reducing terminal group per 26 ± 2 glucose units.

Preparation of 2:3:4:6-tetramethyl D-glucose anilide.

2:3:4:6-Tetramethyl glucose (70 mgm.) was dissolved in absolute alcohol (3 ml.), freshly distilled aniline (0.1 ml.) added, and the mixture boiled under reflux for 2 hours. On cooling in ice, crystals separated, which were recrystallised from dry ether/light petroleum (b.r. 40-60°) (1:1).

Yield, 40 mgm.
M.P., 137-138° alone or admixed with an authentic sample of 2:3:4:6-tetramethyl D-glucose anilide.
Found N = 4.4% OMe = 39.5%.
(Calc. for $C_{16}H_{25}O_5N$, N = 4.5% OMe = 39.9%).

Fraction 1b - trimethyl glucose (0.021 gm.)

Paper chromatography indicated the presence of a single substance (R_G, 0.81) corresponding to 2:3:6-trimethyl glucose. Hydrolysis of the substance with sulphuric acid, and re-examination by paper chromatography did not reveal the presence of any other sugar. Hypoiodite oxidation by the method described on p. 68, indicated that the sugar was 98% pure. Analysis gave the following results:-

n_d^{15} , 1.4767 (c.f. 109).
OMe , 40.8% (calculated, 41.9%).
$[\alpha]_D^{19}$, + 89.5 ⟶ + 68.4° (C = 0.4% in water).

Rotation of fraction 1b in cold methanolic hydrogen chloride.

Fraction 1b (0.019 gm.) was dissolved in methanolic hydrogen chloride (5 ml.; 2%) at room temperature, and the rotation of the solution was observed at intervals, using a 2 dm. tube. In 7 hours it was found that the rotation underwent the following change:-

$[\alpha]_D^{18}$, + 67.1° (initial) ⟶ - 36.9° (final)

Fraction 2, trimethyl glucose (2.679 gm.)

Examination of this fraction by paper chromatography indicated the presence of a single sugar corresponding to 2:3:6-trimethyl glucose. Hypoiodite oxidation by the method described on p. 68 indicated that the sugar was 98% pure.

The fraction partially crystallised on standing. The material (2.5 gm.) was recrystallised twice from dry ether.

Yield of crystalline material, 1.21 gm.
M.P., 115-117° alone or admixed with an authentic sample of 2:3:6-trimethyl D-glucopyranose.
OMe, 41.6% (Calculated, 41.9%).
$[\alpha]_D^{19}$, + 90.1° ⟶ 70.8° (C = 1% in water).

Rotation of 2:3:6-trimethyl glucose in cold methanolic hydrogen chloride solution.

2:3:6-trimethyl glucose (0.1 gm.) was dissolved in methanolic hydrogen chloride solution (10 ml.; 2%) at room temperature, and the rotation was observed at intervals as before (p. 69). In 7 hours it was found that the rotation underwent the following change:-

$[\alpha]_D^{18}$, + 68.4° (initial) ⟶ - 35.0° (final)

Fraction 3, dimethyl glucose (I) (0.140 gm.)

Chromatographic examination indicated the presence of a

single sugar (R_c, 0.57) which corresponded to 2:3-dimethyl glucose. Hydrolysis of a portion of this fraction with sulphuric acid (2%), and re-examination by paper chromatography, did not reveal the presence of any other sugar. Hypoiodite oxidation by the method described on p. 68 indicated that the sugar was 98% pure.

The fraction was obtained as a pale yellow syrup which partially crystallised on standing for 2 weeks in the cold. It was recrystallised once from dry ethyl acetate. The crystals were tiled and washed with dry ethyl acetate. The washings were retained and evaporated.

Yield of crystalline material, 68 mg. (from 130 mg.)
M.P., 84-86°.
OMe, 29.4% (calculated, 29.7%).
$[\alpha]_D^{19}$, + 113.2° ⟶ + 65.1°.

Preparation of 2:3-dimethyl gluconophenylhydrazide, by the method of Evans, Levi, Hawkins and Hibbert (110).

Preparation of 2:3-dimethyl gluconolactone. 2:3-dimethyl glucose (85 mg.) was dissolved in water (5 ml.) and was treated with liquid bromine (1 ml.). The solution was kept at room temperature with occasional shaking for 5 days until it was non-reducing. It was then aerated to remove the bromine and was neutralised with silver carbonate. After removal of the silver chloride by filtration, excess silver was precipitated by hydrogen sulphide and removed. The solution was again aerated to remove the soluble gas and was concentrated to a syrup at 40°/20 mm.. This syrup was transferred to a micro-distillation apparatus and distilled in a high vacuum (bath

temp., 155-160°; 0.005 mm.). A thick syrup was obtained which partially crystallised on standing overnight.

Yield, 67 mg. (80% of theoretical).
OMe, 28.8% (Calculated, 30.1%).

Preparation of 2:3-dimethyl gluconophenylhydrazide.

The 2:3-dimethyl gluconolactone (see p. 92) was dissolved in anhydrous ether (5 ml.) by refluxing for 3 hours. A fine white precipitate formed at once on addition of freshly distilled phenylhydrazine (0.8 gm.), and after 4 hours' refluxing it was removed by filtration. Additional material was recovered from the filtrate by further treatment with phenylhydrazine and concentration of the reaction mixture.

Yield of crude material, 80 mgm. (92% of theoretical). After 2 recrystallisations from ethanol, the material was obtained in the form of short white needles. Yield, 40 mg.

M.P., 160-162°.
Found C, 53.7%; H, 7.1%; N, 9.0%; OCH_3, 19.3%.
Calc. for $C_{14}H_{22}O_6N_2$: C, 53.5%; H, 7.0%; N, 8.9%; OCH_3, 19.7%.

Fraction 4, dimethyl glucose (II) (0.032 gm.)

Paper chromatography indicated that this fraction (R_G, 0.50) consisted of 2:6- or 3:6-dimethyl glucose, or was a mixture of the two. Hydrolysis of a portion of this fraction with sulphuric acid, and re-examination by paper chromatography, did not reveal the presence of any other sugar. Hypoiodite oxidation showed that the dimethyl glucose was 98% pure.

The fraction was obtained as a syrup which failed to crystallise on standing for several weeks in the cold.

OMe, 28.9% (Calculated, 29.7%).
$[\alpha]_D^{18}$, + 78.1° ⟶ + 61.7° (C = 1% in water).

Rotation of fraction 4 in cold methanolic hydrogen chloride solution.

Fraction 4 (0.025 gm.) was dissolved in methanolic hydrogen chloride solution (5 ml.; 2%) at room temperature, and the rotation was observed at intervals using a 2 dm. tube. In 8 hours it was found that the rotation underwent the following change:-

$$[\alpha]_D^{20}, \quad \underset{\text{(initial)}}{+61^\circ} \longrightarrow \underset{\text{(final)}}{-11^\circ}$$

Estimation of 2:6-dimethyl glucose present in fraction 4.

The solution of fraction 4 (0.025 gm.) in methanolic hydrogen chloride (see above) was heated at 100° for 3 hours. The solution was then neutralised and treated with sodium metaperiodate by the method described on p. 71.

It was found that the uptake of sodium metaperiodate was 0.79 moles per dimethyl methyl glucoside unit. This indicated the presence of 79% of 2:6-dimethyl glucose in the fraction.

Estimation of 3:6-dimethyl glucose present in fraction 4.

After estimation of the periodate uptake (see above), the solution was extracted and hydrolysed as described on p. 71. The residue obtained was examined by paper chromatography. The presence of a single substance (R_G, 0.51) corresponding to 3:6-dimethyl glucose was indicated. Quantitative estimation of the substance, using alkaline hypoiodite (see p. 68), showed that 4.83 mg. was present.

Fraction 5.

Chromatographic examination indicated the presence of 2 substances. One (R_G, 0.26) corresponded to monomethyl glucose, whilst the other (R_G, 0.09) corresponded to glucose. The

fraction was not examined further.

PERIODATE OXIDATION OF AMYLOPECTIN.

Estimation of formic acid liberated from the end-groups.

This was carried out by the method described on p. 76 for amylose.

In the case of amylopectin, however, the polysaccharide remained in solution after oxidation with periodate, and was withdrawn when samples were taken for titration. No corrections were, therefore, made to the titration figures as in the case of amylose (p. 77). Results were as follows:-

Wt. of amylopectin, 0.5043 gm. Volume of solution, 120 ml. 20 ml. samples withdrawn.

Time of Shaking (hrs.)	Volume of sodium hydroxide (0.01N) used per 20 ml. sample. (ml.)
160	2.150
180	2.165
250	2.290
325	2.390
Estimated titre after 150 hours' shaking.	2.135 ml.
Yield of formic acid from 162 gm. amylopectin.	0.0412 moles.
Number of glucose residues per non-reducing terminal group.	24

Hydrolysis of amylopectin oxidised by periodate.

Amylopectin (1 gm.) was mixed with sodium metaperiodate (30 ml.; 0.3M), potassium chloride (5 gm.) and water (110 ml.) in a glass-stoppered bottle, and the mixture was shaken at room temperature for 10 days. Excess of ethylene glycol (2.5 ml.) was then added, and shaking was continued for 24 hours. The oxidised amylopectin was dialysed until free from oxidant (test

with potassium iodide and sulphuric acid and diphenylamine and sulphuric acid). The solution was reduced in volume and hydrolysed with sulphuric acid (0.5N; total volume of solution; 200 ml.). The acid was neutralised with barium carbonate and the barium sulphate was filtered off and washed. The filtrate was concentrated and examined by paper chromatography.

Glucose was found to be present. Quantitative estimation by the method of Flood, Hirst and Jones (76), using xylose as a standard, revealed the presence of 0.59 gm. glucose per 100 gm. amylopectin.

PERIODATE OXIDATION OF BARLEY STARCH.

Estimation of formic acid liberated from end-groups.

This was carried out by the method described on p. 76 for amylose. As the oxidised starch granules were not removed from the solution on withdrawal of samples for titration, corrections were applied to the titration figures as in the case of amylose (see p. 76). Results were as follows:-

Wt. of dry starch, 0.5004 gm. Total volume of solution, 120 ml.
20 ml. samples withdrawn.

Time of Shaking (hrs.)	Corrected volumes of sodium hydroxide (0.01N) used per 20 ml. sample. (ml.)
165	1.77
190	1.86
225	2.00
270	2.20
Estimated titre after 150 hours' shaking	1.71 ml.
Yield of formic acid (moles) from 162 gm. amylose	0.033 moles.

Number of non-terminal glucose residues per non-reducing terminal group	30
Calculated number of non-terminal glucose residues per non-reducing terminal group in the amylopectin fraction	24

Hydrolysis of barley starch oxidised by periodate.

This was carried out by the method described on p. 95 for amylopectin, with the following modification:-

After shaking with ethylene glycol, the oxidised starch was filtered on a G3 sintered-glass filter, washed with cold water until free from oxidant (test as before) and then with alcohol, and dried in a vacuum desiccator over calcium chloride. It was then hydrolysed with sulphuric acid (2%) as before.

Chromatographic examination showed the presence of glucose.

Quantitative estimation, as before, revealed the presence of 1.8 gm. glucose per 100 gm. starch.

Attempted determination of the degree of polymerisation of amylopectin.

This was carried out by the method described on p. 79, using 3:5-dinitrosalicylic acid.

Very low and inconcordant "Spekker" drum readings were obtained from the reduced solutions. It was concluded, however, that the degree of polymerisation of amylopectin was greater than 1,500 units corresponding to a minimum molecular weight of 250,000.

The whole starch similarly gave low and inconcordant results.

DISCUSSION.

DISCUSSION.

The barley used in the present investigation was of the "Pioneer" variety, a type commonly used in breweries for malting purposes. It had been dried at a low temperature to a moisture content of 10.7% and had a satisfactory appearance.

The starch was prepared by a method which did not involve the use of substances likely to cause degradation. The yield of starch was 137 gm. from approx. 900 gm. dry barley. As the latter contains about 60% starch (111), the yield was only 25%. However, the barley was not finely ground and starch was sieved out with the fibrous material. A further quantity, associated with protein, was removed during the centrifuging process (see p. 47). Harris and Jesperson (112) treated impure barley starch with sodium hydroxide solution (0.5N) for 20 hours at 5°C to remove the associated protein and thus improve the yield of starch. This treatment, however, was avoided since it may bring about modification of the starch and since it was found exceedingly difficult to free the starch completely from the sodium hydroxide.

The starch was white in colour and when examined under the microscope appeared in the form of granules which were ovoid to spheroidal in shape and were of a moderate size (10-30 μ). It was found that very few of the granules were ruptured, indicating that the milling process had caused no physical degradation of the starch. The starch had low ash (0.2%) and protein (0.26%) contents. Although the fatty acid content of the starch was not determined, it was considered that the extractions with

light petroleum and aqueous methanol had reduced the content to a value which would not prevent the full uptake of iodine during the potentiometric titration experiments. The specific rotation in sodium hydroxide solution (N) was + 157°, which agrees, fairly well, with the values previously recorded for other starches (14,17,19). The specific rotation in perchloric acid (30%) was found to be + 188°. (Perchloric acid has recently been used by Pucher, Leavenworth and Vickory (113) as a solvent for extracting starch from plant tissues, thus facilitating the subsequent quantitative estimation of the starch . The latter in concentrations of less than (0.75%) dissolves easily in the acid, giving clear solutions). Frahn (114) has similarly found that certain wood starches gave higher specific rotations in perchloric acid solution than in sodium hydroxide solution.

The starch had a "Blue Value" of 0.27-0.28. The amylose content, determined by potentiometric titration with iodine, was found to be 18.7%. Thus barley starch appears similar to other cereal starches in amylose content (see Table II).

Hydrolysis of the starch with sulphuric acid, and examination of the product by paper chromatography, indicated the presence of glucose only. Estimation of the glucose by two different methods revealed the presence of 96-97% of the theoretical yield of glucose from the starch. In addition to decomposition during the hydrolysis, the low analytical figures may be due to the presence of small amounts of non-reducing substances (see previous page) and to incomplete drying of the polysaccharide. Since no derivatives, other than those of

glucose, were encountered in subsequent work, it was concluded that the starch was built up from glucose only.

The starch was acetylated by the method of Pacsu and Mullen (105) using acetic anhydride and pyridine. The product was obtained in good yield and had an acetyl content of 43.3% and a specific rotation of + 170°. Viscosimetric measurements showed that the acetylated starch had a high molecular weight. It was calculated that the unsubstituted starch had a molecular weight of 458,000. Barley starch thus shows similarity to other starches in possessing a high molecular weight.

The starch acetate was deacetylated by the method of Zemplen and Pacsu (106) using sodium methylate. The deacetylated starch had a similar "Blue Value" and specific rotation in perchloric acid solution to the original material. It was utilised later in the fractionation experiments.

THE FRACTIONATION OF BARLEY STARCH.

Bourne, Donnison, Haworth and Peat (32) developed their "standard" method for the most favourable precipitation of the amylose fraction of potato starch using thymol, by a systematic variation of many factors, including concentration of starch, viscosity and pH of the starch dispersion, temperature at which precipitation of the amylose-thymol complex is allowed to proceed, etc. This method was applied to barley starch although on a smaller scale (10 gm.) than that employed by Bourne et al. (who generally used samples of 100 gm. or more).

The precipitated fraction was shown, by potentiometric titration with iodine, to contain 60% of amylose. The yield,

however, indicated that the fraction contained only 32% of the total amylose present in the starch. The non-precipitated fraction contained 8% amylose (det. by potentiometric titration) and 75% of the total amylopectin. The undissolved residue obtained on centrifuging the starch paste before the addition of the precipitant was very large (42%). It was possible that either a portion of the amylose fraction had retrograded during the dispersion or simply that the starch was incompletely dispersed. Later experiments, however, seemed to indicate clearly that the dispersion was incomplete.

The "standard" method was applied in three similar experiments (Methods 1B, C and D) with one modification in each case. In the first case (Method 1B), deacetylated starch was used. This was found to disperse much more easily than the granular starch, since only about 2% of undispersed material was obtained. The precipitated fraction contained 66% amylose. The yield indicated that the fraction contained 63% of the amylose present in the starch. It will be noted that in this case, and in all other cases (unless stated otherwise), the amylose and amylopectin contents were calculated by proportion from the "Blue Values." The figures thus obtained must be regarded as only approximate (see p. 15). The non-precipitated fraction contained 7.4% amylose and 89% of the total amylopectin.

The second modification (Method 1C) applied to the "standard" method involved the subjection of the starch paste to a five minutes' high-speed dispersion in an "Ato-mix" disperser. In this case, the amount of undispersed material

was also low (4%). The precipitated fraction contained 74% amylose. The yield of the fraction indicated that it contained 56% of the total amylose. The non-precipitated fraction contained 7.4% amylose and 94% of the total amylopectin.

The third modification (Method 1D) involved the stirring of the starch paste for 3 hours instead of 20 minutes. The amount of undispersed material was again low (4%). The precipitated fraction contained 74% amylose. The yield indicated that the fraction contained 72% of the total amylose. The non-precipitated fraction contained 6.6% amylose and 92% of the total amylopectin.

Thus in the various modifications of the "standard" method, it was found that satisfactory dispersions of the starch were obtained. The second and third modifications indicated that fractionation of the untreated starch was as efficient as that of the deacetylated starch.

Fractionations were also carried out by the methods of Higginbotham and Morrison (47) using butanol (Method 2) and pyridine (Method 3). The butanol-precipitated fraction contained 65% amylose. The yield indicated that the fraction contained 68% of the total amylose. The non-precipitated fraction contained 7.1% amylose and 87% of the total amylopectin. The pyridine-precipitated fraction contained 71% amylose (by potentiometric titration). The yield indicated that the fraction contained 76% of the total amylose. The non-precipitated fraction contained 5.3% amylose and 89% of the total amylopectin.

Thus in all the six fractionations described, the "amylose" fractions contained 25-40% of amylopectin as impurity, and represented (except in one case) 56-76% of the total amylose present in the starch. The "amylopectin" fractions, although they represented (except in one case) more than 86% of the total amylopectin, still contained 5-8% amylose. It therefore appeared that no single method of fractionation was capable of yielding amylose and amylopectin fractions, each with a high degree of purity.

The fractionations using butanol and pyridine together (Methods 4A, B and C) were principally attempted to obtain pure amylopectin fractions. The results of these fractionations will be discussed later (see p. 117).

Reprecipitations were carried out by two different methods in attempts to purify the "amylose" fraction. Firstly, the precipitated fractions prepared by Methods 1C and 1D (B.V. 1.03) were combined and subjected to reprecipitation with thymol under the same conditions as used in the "standard" method. No further purification of the "amylose" fraction took place, as indicated by the slight lowering of the "Blue Value" (to 1.01). An undispersed residue was obtained which seemed to indicate that a portion of the amylose had retrograded during the initial precipitation with thymol. Secondly, the precipitated fraction (amylose content, 65%; B.V., 0.90) prepared by Method 2, using butanol, was subjected to reprecipitation by the same reagent. A precipitated fraction was obtained with an amylose content of 76% (B.V., 1.06) in good yield. The undispersed residue in

this case was very small. Thus the results of the reprecipitations indicated that butanol was more satisfactory than thymol for carrying out purification of the "amylose" fraction.

Although in the initial fractionation experiments, the fraction, precipitated by pyridine (Method 3), was not the purest amylose fraction (see Table VII, p. 57), it contained the greatest proportion (76%) of the total amylose present in the starch. It was decided, therefore, to submit a quantity of starch to fractionation with pyridine, and subject the "amylose" to reprecipitation with butanol.

The precipitated fraction in the larger scale experiment had the same "Blue Value" (0.99) as obtained before. The yield, however, was greater, over 80% of the total amylose being obtained in the fraction. The amylose-pyridine complex was not broken down by trituration with alcohol as usual, but was submitted directly to reprecipitation with butanol. The amylose concentration in the solution was 0.2% as recommended by Higginbotham and Morrison (47). Small samples were withdrawn after each reprecipitation, and the "Blue Values" and iodine uptakes (by potentiometric titration) were determined. After six reprecipitations, it was found that the amylose had an iodine uptake of 20.9% as determined by potentiometric titration. This value was not increased by further reprecipitation of the amylose, even in 0.1% concentration. It compares favourably with the values (21.5%) found by Higginbotham and Morrison (39) for other starches. It was considered, therefore, that the amylose fraction was in the purest condition as determined by

the present methods of analysis. The final yield of the fraction indicated that 80% of the total amylose was present.

The amylose-butanol complex was examined under the microscope. It was found that the structures observed showed similarities to those already described in the literature. After the second reprecipitation, it was found that the complex appeared in the form of six-segmented particles which were similar to those found by Schoch (27) for the corn starch amylose-butanol complex. After the third reprecipitation, the complex took the form of rectangular platelets similar to those found by Kerr (38). After the fourth reprecipitation, the amylose-butanol complex appeared in the form of short needles similar to those found by Kerr (51,73) for the tapioca amylose-butanol complex. The shape of the complex was not altered by further reprecipitation.

Although suggestions (38,51,73) have been put forward about the significance of the different structures, it would appear from the results with barley amylose that its complex with butanol is approaching its purest state when it appears in the form of short needles. The amylose fractions with the highest uptakes of iodine (20.5-20.7% det. by potentiometric titration) found by Kerr (51,73) existed as the complex with butanol in the form of short needles also. It is unfortunate that the crystalline appearance of the pure amylose-butanol complexes obtained by Higginbotham and Morrison (see p. 13) have not been described.

The amylose-butanol complex dissolved easily in warm water.

The amylose, however, retrograded from solution in a similar manner to the amyloses from other starches. It has been recognised that the complex with butanol is stable (47,49), and the bulk of the amylose was stored in this form in water saturated with butanol. Solutions for analysis were freshly prepared by dissolving the complex directly rather than by isolating the amylose first. The concentration was then found by evaporating an aliquot of the solution to dryness and weighing directly. In this way it was possible to overcome, almost completely, the disadvantages of retrogradation.

The amylose had a specific rotation of + 205° in water, + 200° in perchloric acid (30%), and + 149° in NaOH (see p. 99). Hydrolysis of the amylose with sulphuric acid and estimation of the resultant glucose by oxidation with alkaline hypoiodite gave 97.9% of the theoretical yield. This value agreed very well with those found after hydrolysis of the whole starch (see p. 99).

The major part of the investigation was concerned with the classical technique of methylation and the quantitative estimation of the derived methylated glucoses. The amylose was methylated by treatment with sodium hydroxide and dimethyl sulphate in an atmosphere of nitrogen. After 14 treatments with the reagents, a product was obtained, the methoxyl content (44.7%) of which was not raised on further treatment. The methylated amylose, which was obtained in 67% yield, was fractionated by precipitation from chloroform with light petroleum. Two fractions (A and B) were obtained and examined separately. Fraction A had $[\alpha]_{D,}$ + 206°; OMe, 44.8% and

$\frac{\eta_{sp}}{c}$, 0.45 in m-cresol. Fraction B had $[\alpha]_D$ + 204°; OMe, 44.9% and $\frac{\eta_{sp}}{c}$, 0.335 in m-cresol. No constants for the Staudinger equation have, so far, been calculated for methylated amylose dissolved in m-cresol. It was considered possible, however, to obtain very approximate values for the degree of polymerisation by using the constant for the structurally similar methylated cellulose. This has been found by Staudinger and Husemann (90) to be $K_m = 10 \times 10^{-4}$. Using this value, it was calculated that fraction A had a degree of polymerisation of 450 glucose units, whereas fraction B had a degree of polymerisation of 330 units. The significance of these figures will be discussed later (see p. 114).

Small portions of both fractions were hydrolysed and the mixtures of methylated sugars were examined by paper chromatography (77). In fraction A the presence of (a) 2:3:6-trimethyl glucose in quantity, and (b) dimethyl glucose in smaller concentration, was indicated. In fraction B the presence of (a) 2:3:4:6-tetramethyl glucose, a trace, and (b) 2:3:6-trimethyl glucose in quantity, was indicated. The sugars were identified by measuring their R_G values (77) and confirming by running standard sugars against the hydrolysate. The proportions of tetramethyl and dimethyl glucoses in fractions B and A, respectively, were so small as compared with the proportion of trimethyl glucose that quantitative estimation by the method of Hough, Hirst and Jones (77) was considered impracticable. Fraction A was then hydrolysed to glycosides and to free sugars. The mixture of methylated sugars was separated by the method of

Hough, Jones and Wadman (78) using a cellulose column. The column was eluted with a light petroleum-n-butanol mixture (1:1) saturated with water, and the eluate was collected in a series of tubes. Examination of the contents of every tenth tube revealed the position of the 2:3:6-trimethyl glucose in the eluate, but did not reveal the presence of any other sugar. The combination and concentration of the contents of groups of five tubes revealed the presence of the 2:3:4:6-tetramethyl glucose. The dimethyl glucoses, together with traces of monomethyl glucose and glucose, were not eluted with the solvent and were obtained by washing the column with water. Three fractions were thus obtained and the total recovery was over 85%.

The tetramethyl glucose fraction (0.022 gm.) accounted for approx. 1% of the sugars recovered from the column (2.278 gm.). Hydrolysis of a portion of this fraction, however, indicated that it contained trimethyl methylglucoside as impurity. Connell (115) has similarly found that trimethyl methylglucoside appeared with the tetramethyl glucose fraction from hydrolysed methylated laminarin, whilst Chanda (116) found that dimethyl methylxyloside appeared with the trimethyl xylose fraction from hydrolysed methylated xylan. It is probable that the non-removal of methanol as a hydrolytic product prevents completion of the formation of free sugars according to the following equation:-

$$C_9H_{17}O_5(OCH_3) + H_2O \underset{\leftarrow}{\overset{H^+}{\rightleftharpoons}} C_9H_{18}O_6 + CH_3OH.$$

Quantitative estimation of the mixture by the method of Hirst, Hough and Jones (77) indicated the presence of 33.7% of

tetramethyl glucose. This corresponds to the presence of one non-reducing terminal group per 370 ± 30 glucose residues. (In this and in later cases in determining the proportion of "end-group," the higher figure was obtained by calculation from the weight of free sugars before separation, whilst the lower figure was obtained from the total weight of sugar recovered after separation).

As the quantity of tetramethyl glucose was, therefore, indicated to be very small, it was not possible to authenticate it fully. The quantity present was estimated directly by oxidation with alkaline hypoiodite. This oxidation and all oxidations by the method described by Hirst, Hough and Jones (77) were carried out using a sodium hydroxide-phosphate buffer (pH, 11.4) (108) instead of the carbonate-bicarbonate buffer (pH, 10.6) (77), as the former had the distinct advantage of there being less chance of losing any iodine during effervescence while acidifying the solution. This reagent has been found (116) to oxidise stoichiometrically the common aldoses and their methylated derivatives. Two results were obtained which showed that the fraction contained a total of 6.55 ± 0.15 mgm. tetramethyl glucose. This corresponds to the presence of one non-reducing terminal group per 400 ± 40 glucose residues. This value shows good agreement with that calculated above, since the error in calculating the amount of tetramethyl glucose in the first case is ± 8% (77).

The yield of the "end-group" or the partially methylated sugars was not seriously affected, due to demethylation during

the acid hydrolysis. In experiments carried out under almost the same conditions as used in the large scale hydrolyses, chromatographically pure 2:3:4:6-tetramethyl glucose was converted to trimethyl glucose to the extent of 1%. This result does not significantly affect the "end-group" calculations made above. Chromatographically pure 2:3:6-trimethyl glucose was demethylated by 0.7% to dimethyl glucoses.

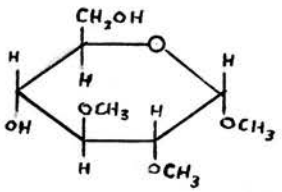
After oxidation and estimation of the tetramethyl glucose, the solutions were retained and the trimethyl methylglucoside was isolated, hydrolysed and estimated. 12.95 mgm. trimethyl glucose corresponding to 13.77 mgm. of the methylglucoside was found to be present. After considering the losses which occurred during the extra manipulations required to isolate the trimethyl methylglucoside, it was concluded that the fraction contained only the two sugars mentioned above.

Fraction 2 was shown by hypoiodite oxidation to consist of almost pure trimethyl hexose. It was obtained in crystalline form and corresponded with 2:3:6-trimethyl D-glucopyranose in its physical and chemical properties. This was confirmed by its inversion of optical rotation in cold methanolic hydrogen chloride solution (109,117).

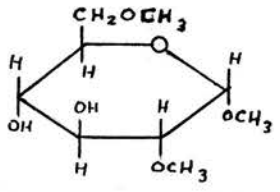
In fraction 3, the presence of the following sugars was indicated by paper chromatography:- (a) 2:3-dimethyl glucose, (b) 2:6- or 3:6-dimethyl glucoses or a mixture of the two. Both isomers have the same R_G value and are not separated by paper chromatography. Monomethyl glucose and glucose were present as traces. Quantitative estimation indicated the

presence of 2.6% of 2:3-dimethyl glucose (equivalent to 1.02 mgm.) in the mixture. The fraction exhibited inversion of the optical rotation in cold methanolic hydrogen chloride solution in a similar manner to 2:3:6-trimethyl glucose as above. This indicated that position 4 in the sugars present in the fraction was unsubstituted to a large extent since it is believed that the inversion arises through the formation of the methylglucofuranosides. This confirmed the indications already revealed by paper chromatography that the fraction also contained 2:6- and/or 3:6-dimethyl glucoses. No satisfactory method has, as yet, been developed for separating the two isomers. Bell (80), however, has recently developed a method which enables the proportions of the two in mixtures to be estimated even in the presence of 2:3-dimethyl glucose. The method is based on the reaction of the dimethyl methylglucopyranosides with sodium metaperiodate (see below).

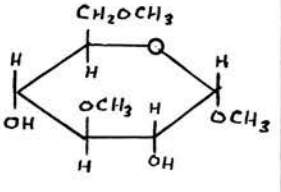
On heating the methanolic hydrogen chloride solution for several hours at 100°C, the methylglucopyranosides were formed. Inspection of fig. XIX



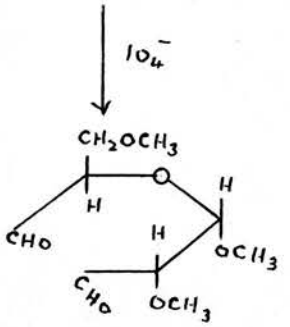
2:3-dimethyl methylglucopyranoside



2:6-dimethyl methylglucopyranoside



3:6-dimethyl methylglucopyranoside



(FIG. XIX)

will show that if the 2:3-, 2:6- and 3:6- derivatives are present in the mixture, then only the 2:6- derivative would react with sodium metaperiodate since it possesses a pair of contiguous hydroxyl groups on C_3 and C_4 (see p. 38). One molecule of the reagent would be utilised per molecule of 2:6-dimethyl methyl-glucoside. Since the uptake of periodate was 0.84 moles per dimethyl methylglucoside unit, it was concluded that the fraction contained 84% 2:6-dimethyl glucose. After treatment with periodate, the solution was extracted to obtain the unattacked dimethyl methylglucosides. After hydrolysis, the presence of the 3:6-dimethyl glucose was indicated by paper chromatography along with the 2:3-dimethyl glucose. The significance of this dimethyl glucose fraction will be discussed later.

Fraction B was examined in exactly the same way as fraction A. After hydrolysis and separation of the hydrolysate on a cellulose column, three fractions were again obtained.

The tetramethyl glucose fraction (0.010 gm.) contained trimethyl methylglucoside as impurity. After hydrolysis, the presence of 39.6% tetramethyl glucose was found in the mixture, corresponding to the presence of one non-reducing terminal group per 185 ± 15 glucose units. The quantity of tetramethyl glucose was determined directly by hypoiodite oxidation and found to be 3.39 ± 0.09 mgm. This corresponded to the presence of one non-reducing terminal group per 215 ± 25 glucose units which compares favourably with the result above. The amount of trimethyl methylglucoside, present as impurity, was again

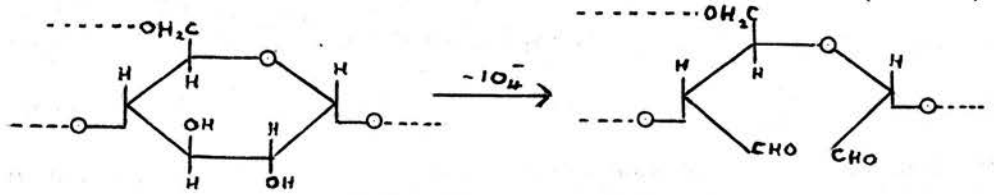
calculated, and it was concluded that the fraction contained only the two sugars mentioned.

The trimethyl glucose fraction was again obtained in crystalline form and corresponded to 2:3:6-trimethyl D-gluco-pyranose. It was characterised by its inversion of optical rotation in cold methanolic hydrogen chloride solution.

The dimethyl fraction was also very small and the components were estimated by the method of Hirst, Hough and Jones (77) using pure 2:3:6-trimethyl glucose as a reference sugar. 0.70 mgm. 2:3-dimethyl glucose and 1.31 mgm. 2:6- and/or 3:6-dimethyl glucoseswere found to be present.

Now it was found that, after hydrolysis of the amylose fully oxidised by periodate, no glucose was observed on examination by paper chromatography. This indicated that there were no glucose residues in the amylose which were unattacked by periodate. Thus all the glucose residues were joined together only through C_1, C_4, or C_6 (fig. XX) and none through C_2 or C_3 (see p. 44).

(FIG. XX)



The 2:6- and 3:6-dimethyl glucoses present in the methylated amylose fractions must, therefore, have arisen through demethylation and incomplete methylation of the polysaccharide, and not through their existence as "branching points". (Since the amount of demethylation of 2:3:6-trimethyl glucose was 0.7%, it accounted for not more than 40% of the "extra" dimethyl

glucose).

It was possible, however, that the 2:3-dimethyl glucose arose through the presence of a branching in the 6-position of a glucose unit (see fig. XX). If there was only one branching point in the chain, then the hydrolysed methylated material would contain tetramethyl glucose and 2:3-dimethyl glucose in the molar ratio of 2:1 respectively. If there were two branchings in the chain, the molar ratio would be 3:2, etc. Inspection of the respective molar concentrations of tetramethyl glucose and 2:3-dimethyl glucose (Table VIII) in fraction A indicated that over half of the fraction did not contain any branchings. Since it was found that 2:3-dimethyl glucose was also obtained on the

TABLE VIII.

	Fraction A		Fraction B	
	Moles per cent. in hydrolysate ($\times 10^3$)	Moles/mole tetramethyl glucose	Moles/cent. in hydrolysate ($\times 10^3$)	Moles/mole tetramethyl glucose
Tetramethyl glucose	1.22	1	2.27	1
2:3-dimethyl glucose	0.22	0.18	0.47	0.21

demethylation of 2:3:6-trimethyl glucose (see p. 76), it was possible that the whole amylose fraction possessed no branching points, and consisted of straight chains of about 400 glucose residues joined by 1:4-α-linkages. The viscosmetric measurements (see p.106) and the high optical rotation tend to support this view.

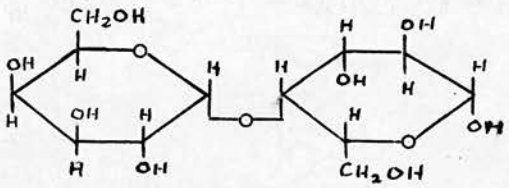
The results for fraction B (Table VIII) indicated that it, too, possessed few, if any, branching points. No other

conclusion, however, may be drawn from fraction B since viscosimetric measurements showed that it had a lower molecular weight than fraction A and may, therefore, have been degraded or contaminated with substances of low molecular weight.

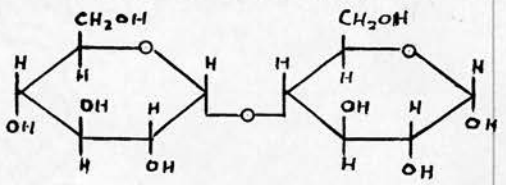
The suggested structure for the amylose did not appear in conformity with the chain length determined from the estimation of the formic acid liberated from the end-groups by periodate oxidation. As described earlier (p. 43), a non-reducing end-group will yield one molecule of formic acid, and a reducing end-group two molecules of formic acid after 150 hours, the time found by Halsall, Hirst and Jones (118), necessary to oxidise completely the structurally similar β-methylmaltoside under the same conditions. Two estimations were carried out and, although the results did not agree closely, the yield of one molecule formic acid per 71 ± 7 glucose residues was indicated. This corresponded to the presence of 215 ± 20 glucose units in an assumed straight chain polymer, or to 290± 30 units if the molecule possessed one branching point. Neither result agreed with the methylation results, and no satisfactory explanation can, as yet, be advanced to interpret the apparent high yields of formic acid. Insufficient amylose remained to permit further study of this reaction.

Estimation of the degree of polymerisation by Meyer's method (p. 79) using 3:5-dinitrosalicylic acid indicated that the amylose molecule contained 135 ± 20 glucose units. This result also did not appear in conformity with the methylation results and was lower than the result from periodate oxidation studies

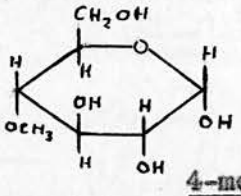
(see previous page). In an attempt to verify its validity, the reaction of maltose, which was used as the model substance of known polymeric size, with the dinitrosalicylic acid, was compared with that of lactose and 4-methyl glucose. Theoretically the molecular reducing power of lactose and maltose (fig. XXI), which are both 4-substituted glucose derivatives, should be equal and should be slightly more than half that of 4-methyl glucose. If the reaction occurred between the reducing group



lactose



maltose



4-methyl glucose.

(fig. XXI)

of the polysaccharide and 3:5-dinitrosalicylic acid as suggested by Meyer, then lactose and maltose should show similar reductions which should be correspondingly lower than that of 4-methyl glucose. The experimental results, however, as judged from the "Spekker" absorptiometer readings, did not agree with this, and suggested that the reaction was not simply between the reducing group and 3:5-dinitrosalicylic acid. . The fact that Meyer found that glucose itself was unsatisfactory for preparing the standardisation curve tended to support this suggestion. The results for the degree of polymerisation of the amylose obtained by this method must, therefore, be treated with reserve until the mechanism of the whole reaction is fully

explained.

From the results, it seems justifiable to consider that barley amylose consists for the most part of straight chains of about 400 glucose residues, joined together by 1:4-α-linkages. It is possible that a portion of the amylose is slightly branched. The linkages at the branching points would be of the 1:6- variety.

The "amylopectin" fraction.

The fractionation methods employed (1,2,3; see pp. 52-55) did not yield an "amylopectin" fraction which contained less than 5% amylose. The subjection of these fractions to further treatment with the precipitants yielded no significant amounts of the amylose complex. The method of Higginbotham and Morrison, using pyridine and butanol together (see p. 26) was then applied. If the starch paste was stirred for a half-hour (Method 4A, p. 55), the "amylopectin" fraction was found to contain 2.8% amylose. After 1 and 3 hours' stirring (Methods 4B and C), the "amylopectin" isolated contained 2% amylose (det. by potentiometric titration.) The fractions in each case contained about 50% of the total amylopectin present in the starch. A quantity of starch was, therefore, fractionated by Method 4B and the amylopectin was obtained in similar yield and containing 2% amylose as above.

The results of the fractionations seemed to indicate that in barley starch, as in other starches, there existed a portion of the amylose which was not readily precipitated along with the bulk of the fraction. Fractionation by Method 4 indicated that

a portion of this amylose could be precipitated, but only if a substantial proportion of the amylopectin was carried down with it (see Table VII). These inferences added force to the suggestion made by Higginbotham and Morrison (39) that part of the amylose is linked directly to the amylopectin fraction, thus preventing its precipitation and separation.

The amylopectin gave a reddish-purple coloration with iodine, similar to that from other amylopectins. The specific rotation in perchloric acid (30%) was + 168°, and in sodium hydroxide solution (N), + 149°.

The amylopectin was methylated by repeated treatment with sodium hydroxide and dimethyl sulphate. The initial reaction was much brisker than that with the amylose, and required cooling to prevent a rise in temperature taking place. After 6 treatments with the reagents, a product was obtained in 64% yield, having a methoxyl content of 43.9%. This product was fractionated by precipitation from a chloroform solution with light petroleum. Two fractions (A and B) were obtained, each with a similar methoxyl content (44.0%) and having a specific rotation of + 204° in chloroform. Viscosimetric measurements showed (1) that fraction A had a high apparent molecular weight (520,000, which agreed reasonably well with that calculated from the acetylated starch (see p. 51) and indicated that little, if any, degradation of this fraction occurred during the methylation process), and (2) that fraction B, with a lower apparent molecular weight than fraction A, was probably degraded or contaminated with products of low molecular weight.

Hydrolysis of a small portion and examination of the products from the methylated fraction A indicated the presence of the following:- (a) 2:3:4:6-tetramethyl glucose (4.1%); (b) 2:3:6-trimethyl glucose (81.8%); (c) dimethyl glucoses (12.8%, containing 2:3- (about $\frac{1}{3}$ of total by visual inspection) and 2:6- and/or 3:6-dimethyl glucoses); (d) monomethyl glucose (1.2%); and (e) glucose (a trace). Thus, from the quantities of dimethyl and monomethyl glucoses obtained and from the methoxyl value, it was indicated that the fraction was incompletely methylated. It was treated three times with Purdie's reagents and a product was obtained, the methoxyl content of which (45.0%) was not increased on further methylation. This product was found to be essentially homogeneous. Viscosimetric measurements indicated that the fraction had suffered no degradation during this methylation process.

Hydrolysis of a small portion and examination of the products of the more fully methylated product indicated the presence of the following:- (a) 2:3:4:6-tetramethyl glucose (4.2%); (b) 2:3:6-trimethyl glucose (90.5%); (c) dimethyl glucoses (principally 2:3-dimethyl glucose) (5.3%); and (d) faint traces of monomethyl glucose and glucose. The proportion of tetramethyl glucose (4.2%) corresponds to the presence of one non-reducing terminal group per 25 glucose residues.

The fully methylated amylopectin, in quantity, was hydrolysed to glycosides and then to free sugars. The mixture of methylated sugars was separated on the cellulose column, using light petroleum-n-butanol (1:1) saturated with water as the

solvent. The eluate was again collected in a series of tubes. Examination of the contents of every tenth tube revealed the presence of four fractions (each of which was separated from the others) thus: (1) tetramethyl glucose, (2) trimethyl glucose, (3) dimethyl glucose (I), (4) dimethyl glucose (II). The recovery of sugars was over 92%. On washing the column with water, and evaporating the extract to dryness, a small residue was obtained. This was examined by paper chromatography, and the presence of monomethyl glucose and glucose was indicated in low concentration. It was considered that these substances had no structural significance and resulted from demethylation or incomplete methylation of the polysaccharide.

Hypoiodite oxidation of the tetramethyl glucose fraction (0.152 gm.) indicated that it contained 84.9% of tetramethyl glucose. A portion was hydrolysed and examined by paper chromatography. As in the case of the methylated amylose (see p.108), the fraction was found to be contaminated with trimethyl methylglucoside. It was, therefore, hydrolysed in quantity and the hydrolysate was fractionated on the semi-micro scale on a cellulose column. Two fractions were obtained thus: (Ia) tetramethyl glucose, and (Ib) trimethyl glucose.

Hypoiodite oxidation now showed that fraction Ia consisted of almost pure tetramethyl hexose. It was obtained in crystalline form and corresponded with 2:3:4:6-tetramethyl D-glucose. This was confirmed by forming the crystalline anilide.

Fraction (Ib) was not obtained in crystalline form.

Analysis indicated that it probably consisted of 2:3:6-trimethyl glucose. This was corroborated by its inversion of the optical rotation in cold methanolic hydrogen chloride solution (see p.110).

Fractions 2 and 3 were obtained in crystalline form. Fraction 2 corresponded in its physical and chemical properties to 2:3:6-trimethyl D-glucopyranose. This was confirmed by its inversion of optical rotation in cold methanolic hydrogen chloride solution as above. Fraction 3 corresponded in its properties to 2:3-dimethyl D-glucopyranose. It was authenticated by forming the crystalline 2:3-dimethyl gluconophenylhydrazide which melted at 160-162°(Evans, Levi, Hawkins & Hibbert (110) found 166.5°-167°) and gave good analytical figures.

Fraction 4, dimethyl glucose (II) (0.032 gm.), failed to crystallise even after standing for a long period in the cold. Paper chromatography indicated that the fraction contained 2:6- and/or 3:6-dimethyl glucoses. This was confirmed by the inversion of the optical rotation in methanolic hydrogen chloride solution (see p. 111). Periodate oxidation studies indicated that the fraction contained 79% of the 2:6-isomer. This was confirmed by isolating the sugar remaining after oxidation. Estimation showed that it amounted to about 20% of the fraction and corresponded to 3:6-dimethyl glucose on examination by paper chromatography.

The amount of "end-group" obtained from the quantitative separation of the methylated amylopectin hydrolysate on the cellulose column was indicated to be 129 mgm. which corresponds to the presence of one non-reducing terminal group per 26± 2

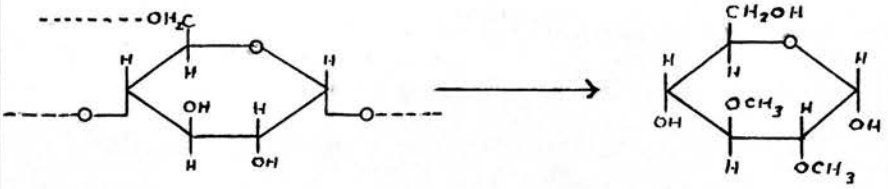
glucose residues. This result agrees with that calculated earlier (p. 119) and indicates that barley amylopectin is similar to other amylopectins in possessing one non-reducing terminal group per 20-26 glucose residues. The molecular weight determined by viscosimetric measurements was found to be high, and it was, therefore, concluded that the amylopectin possesses a highly branched structure.

The molar proportion of dimethyl glucose in the hydrolysate of the methylated amylopectin was greater than that of tetramethyl glucose (see Table IX). Demethylation of 2:3:6-trimethyl

TABLE IX.

	Moles per cent. in hydrolysate (x 10^2)	Moles/mole tetramethyl glucose
Tetramethyl glucose	1.82	1
2:3-dimethyl glucose	2.24	1.23
2:6 / 3:6 -dimethyl glucose	0.51	0.28

glucose (0.7%) would only account for a small proportion of the dimethyl glucoses. As the methoxyl content of the polysaccharide (45.0%) was close to the theoretical value (45.6%) for complete methylation, it was considered that the greater part of the dimethyl glucoses was derived from branching points (see fig. XXII). Thus it may be considered that if the 2:3-



"branching point" 2:3-dimethyl glucose

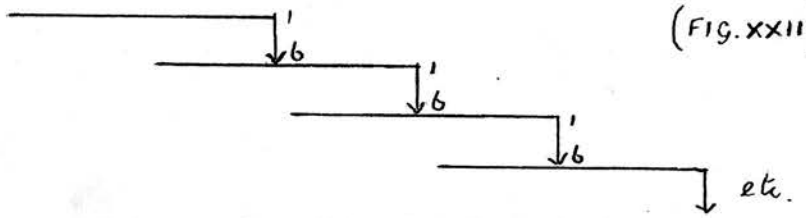
(fig. XXII)

dimethylglucose and the mixture of 2:6-and 3:6 - isomers arise

in the same proportion through "undermethylation", then it may be calculated (see Table IX) that as 81% of the mixture consists of 2:3-dimethyl glucose, then 81% of the "inter-unit" linkages will be 1:6- linkages. If, as appears probable from the results on the methylated amylose fraction A (see p.113), the 2:6- and 3:6-dimethyl glucoses are more likely to occur through "undermethylation" than the 2:3- isomer, even more than 81% of the "inter-unit" linkages must be of the 1:6- variety.

A so-called "laminated" structure may, therefore, be suggested tentatively for barley amylopectin, consisting of a number of "repeating units", each unit being a straight chain of about 26 glucose residues joined by 1:4-α- linkages. The "repeating units" are joined together principally by 1:6-linkages (fig. XXIII).

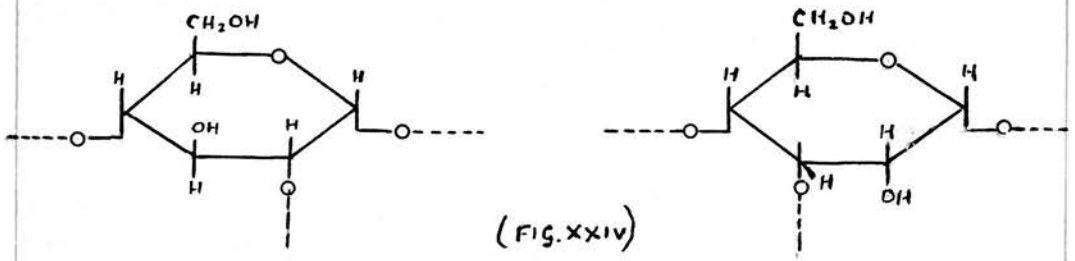
(FIG. XXIII)



This suggested amylopectin structure is in agreement with the results obtained from the oxidation of the polysaccharide with potassium periodate. Estimation of the formic acid liberated from the end-groups indicated the presence of one non-reducing terminal group per 24 glucose residues. (The presence of 2% amylose in the fraction does not significantly alter this figure, if allowance is made for it). Estimation of the formic acid liberated from the end-group in the whole starch indicated the presence of one non-reducing terminal group per 30 glucose residues. By calculation from this value (see

p. 42), it was also indicated that the amylopectin contained 24 glucose residues per non-reducing terminal group.

Hydrolysis of the amylopectin oxidised by periodate, and examination of the product by paper chromatography, indicated the presence of unoxidised glucose residues. Now, if it is first assumed that the oxidation was complete, then if all the "inter-unit" linkages present in the amylopectin were 1:6-linkages (see fig. XX, p. 113), no glucose would be found in the hydrolysate. If all the "inter-unit" linkages were of the 1:2 or 1:3 variety (fig. XXIV), then the polysaccharide would



(FIG. XXIV)

1:2-LINKAGE 1:3-LINKAGE

contain 1 residue in 26, on the basis of the "laminated" structure, which was not oxidised by periodate. Thus 4.27 gm. glucose would be found per 100 gm. amylopectin. Since the yield of unoxidised glucose was 0.59 gm. per 100 gm. amylopectin, it was concluded that over 86% of the "inter-unit" linkages in the amylopectin are 1:6- linkages. This value agrees reasonably well with that indicated from methylation studies (see p. 121). Since it was not possible to decide exactly whether the polysaccharide was completely oxidised by periodate, it is not possible to draw any concrete conclusions regarding the identity of the remainder of the "inter-unit" linkages.

This result also indicated that a greater proportion of

the "inter-unit" linkages in barley amylopectin are of the 1:6-variety than is indicated for other starches. In potato, sago, acorn and waxy maize starches, Halsall, Hirst, Jones & Roudier (14,100) found that over 75% of the linkages in the amylopectins were of the 1:6- variety (see p. 44). In the case of barley, on hydrolysingthe whole starch oxidised by periodate, it was calculated that only about 50% of the "inter-unit" linkages were of the 1:6- variety. Thus it would appear from this result that steric hindrance in the closely packed granular structure probably prevents full oxidation by the periodate and accounts for the divergency between the results for the barley amylopectins and the other amylopectins.

The amylopectin (and the whole starch) possessing few reducing groups failed to act significantly with 3:5-dinitro-salicylic acid. It could be inferred, however, that the amylopectin had a molecular weight in excess of 250,000.

From the above considerations, it seems justifiable to assign a so-called "laminated" structure (see figs. VI and XXIII) for barley amylopectin, consisting of a number of "repeating units" joined by 1:4-α-linkages. Over 86% of the "repeating units"are joined together by 1:6-linkages. It is not possible to decide, at present, how the remainder of the "repeating units" are linked.

SUMMARY.

(1) Barley starch was found to contain 18.7% amylose.

(2) Fractionation of the starch was carried out by different methods, using thymol, pyridine and butanol. In no case was complete fractionation obtained. The precipitated products generally contained 60-75% amylose corresponding to 56-76% of the total amylose present in the starch. The non-precipitated fractions contained 5-8% amylose and more than 86% of the total amylopectin present in the starch.

(3) The highest yield of amylosein the precipitated fraction was obtained by precipitation with pyridine. A quantity of starch was fractionated by this method and the precipitated fraction was purified by reprecipitation with butanol. A fraction was finally obtained (representing 80% of the total amylose present in the starch) which had an iodine uptake of 20.9% and was considered, by the present methods of analysis, to be pure amylose.

(4) Methylation of this fraction yielded a highly methylated product which, after hydrolysis and separation on a cellulose column, yielded 2:3:6-trimethyl glucose in quantity and 2:3:4:6-tetramethyl glucose and dimethyl glucoses in low concentration.

(5) From a study of the methylated products, it was concluded that the amylose consisted, for the greater part, of

straight chains of about 400 glucose residues united by 1:4-α-linkages. It was possible that a portion of the amylose was slightly branched, the "branching" linkage being of the 1:6- variety.

(6) The yield of formic acid obtained after oxidation of the amylose with periodate indicated a much lower chain length than that from methylation studies. No explanation could be forwarded to account for this.

(7) "Amylopectin" fractions were obtained by precipitation with pyridine and butanol used together. These contained 2% amylose and 50% of the total amylopectin present in the starch. A quantity of the "amylopectin" was prepared.

(8) Methylation of the "amylopectin" yielded a highly methylated product with an apparent molecular weight of 520,000. Hydrolysis of this product and separation of the derived methylated glucoses on a cellulose column yielded 2:3:4:6-tetramethyl glucose, 2:3:6-trimethyl glucose, and 2:3-, 2:6- and 3:6-dimethyl glucoses. The amount of "end-group" indicated the presence of one non-reducing terminal group per 26 $\pm$ 2 glucose residues.

(9) From a study of the methylated products, a so-called "laminated" structure is proposed for barley amylopectin, consisting of "repeating units" of about 26 glucose residues, joined together by 1:4-α-linkages. The

"repeating units" are joined principally by 1:6-linkages to form a molecule with a high molecular weight.

(10) Periodate oxidation studies confirmed the results obtained by methylation by indicating the presence of one non-reducing terminal group per 24 glucose residues, both from experiments with the amylopectin and with the whole starch. Hydrolysis of the amylopectin oxidised by periodate and estimation of the derived glucose indicated that over 86% of the "repeating units" were joined to each other by 1:6 linkages. No conclusion could be drawn about the remainder of the linkages.

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